SOME FACTORS DETERMINING THE LOCALIZATION OF A CHICKEN TUMOR AGENT.*

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PLATES 19 AND 20.

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In the production of many pathological processes, two main factors are concerned. The first, the general factor, which determines the nature of the condition, and the second, or local factor, determining the site of the inflammatory process.

In osteomyelitis for example, there is frequently the history of an antecedent injury to the affected part, and it may be taken for granted, that this injury in some way predisposes to a localization of bacteria, which may happen to be present in the circulating blood or elsewhere. In syphilis, tertiary lesions appear more frequently in superficial bones which are more exposed to injury than in the deeper ones which are well protected by overlying tissues. Kiedel and Zimmermann (1) have described an interesting case, showing that under certain conditions a syphilitic eruption may follow the lines of tattoo markings in the skin, and further, that certain of the pigments used in tattooing are more active than others in this relation, though whether because of a specific action of the dye substance, or of the degree of reaction that it induces is not known. That all inflammatory reactions are not equally effective in promoting the growth of organisms, is well shown by the work of Chesney and Kemp (2), who have demonstrated, that spirochetes will survive better and multiply more rapidly in a recent granulating wound, than in an older subacute lesion.

Various attempts have been made to study the question of localization experimentally.

Thus Gye and Kettle (3) have demonstrated the localizing effect of silica in tuberculosis. Levaditi and Nicolau (4) have shown that the trauma produced by the removal of hair is sufficient to determine a localization of the eruption,

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when vaccine virus is injected intravenously. Somewhat similar results may be obtained when the "virus" of epithelioma contagiosum is injected intravenously in birds, the lesions developing in this case around the roots of feathers which have recently been plucked (5).

Rous and Murphy and Tytler (6) while working with the filtrates of those chicken tumors, which can be transmitted by means of a cell-free filtrate, found that a larger number of tumors could be obtained, if a certain amount of tissue derangement was caused at the site of the injection. To produce this they added kieselguhr to the inoculum. This substance has been shown by Podwyssozki (7) to produce a well marked tissue reaction when injected subcutaneously. Jones and Rous found that injury determined the localization of the growths developing after the inoculation of mouse tumor material into the peritoneal cavity (8).

Metastasis formation by "filterable chicken tumors" is common, probably because, as shown by Rous, Robertson and Oliver (9), the active agent is frequently present in the blood of chickens with developing tumors. The secondary growths may occur in the liver, lungs, etc., but during the spring season they are much more common in the ovary. It has been suggested, that this seasonal infection of the ovary is due to the frequent injuries received by that organ during the process of ovulation, and the resulting presence of much recent granulation tissue, which it seems is readily attacked by the tumor agent. Rous, Murphy and Tytler (6) investigated the action of the tumor agent when injected intravenously in chickens, and found that tumors seldom resulted, but when they did develop it was nearly always in the functioning ovary.

The above observations on chicken tumors suggested that the induced inflammatory reactions might prove favorable to the localization of the causative agents on intravenous injection with as a result the subsequent development of tumors. It was therefore determined to attempt the experiment.

Method.

The method selected was the injection into the pectoral muscles of various irritating substances such as Scharlach R, kieselguhr, tar and hashed chicken embryo tissue, followed after a period by the intravenous injection of fresh tumor filtrate. The pectoral region was selected as the site, partly for convenience, and partly because metastases have never been reported there, even after intravenous injection of the tumor agent. Chicken Sarcoma 1 of the series described from The Rockefeller Institute was chosen for use in these experiments, and only adult Plymouth Rock and Rhode Island red hens were used. These birds are now equally susceptible to this tumor.

Experiments with Scharlach R.

First Experiment.—Six hens received 1 cc. of a saturated solution of Scharlach R in olive oil in each breast and this injection was repeated after 7 days. There resulted a certain amount of inflammatory reaction which could be made out on palpation. Three weeks after the last injection of Scharlach R each fowl received 5 cc. of fresh tumor filtrate into the wing vein. This filtrate was prepared in the usual way by grinding about 25 gm. of the tumor material with sand and about 400 cc. of Ringer's solution, centrifuging to remove the larger particles and filtering through a Berkefeld V filter.

Four weeks later the fowls were killed and thoroughly examined. The pectoral muscles showed a marked inflammatory reaction around the Scharlach R which had become localized and was lying in pockets shut off by well formed connective tissue. Only in one bird out of the five was a tumor present in the breast tissue, this being in contact with the reaction tissue. That this failure to localize in the pectoral tissue was not due to general resistance of the chickens or to the low potency of the filtrate was clearly demonstrated by the fact that four of the five animals showed formations of tumors in other locations, namely lungs, spleen, ovaries, liver and in the wing at the site of the filtrate injection.

In only one fowl out of six employed in this experiment a tumor developed in the breast muscle in the region infiltrated with Scharlach R. In view of this fact the procedure was slightly altered, the period between the second injection of Scharlach R and the intravenous injection of tissue filtrate being shortened.

Second Experiment.—Five hens were injected into the breast muscle with the same solution of Scharlach R as before. Four of these received 1 cc. on each side, while the last received only 0.5 cc. After an interval of 7 days these injections were repeated. A week later, the intravenous injection of 5 cc. of fresh tumor filtrate prepared as described above was made into a wing vein. The animals were killed 4 weeks later, and the breast tissue was examined.

As before, the solution of Scharlach R spread through the pectoral tissue and became only slightly encapsulated by fine connective tissue. In all these birds, small tumor nodules were found in the breast muscles, lying amongst the Scharlach R (Fig. 1). This was later confirmed by microscopical examination. The smaller amount of the dye received by one of the hens did not appear to affect the result, which was the same in all the injected fowls.

Experiments with Tar.

Following the same plan as before, three fowls were injected with 1 cc. of a solution of purified tar in benzene. This was repeated after a week. Seven days later they were given an intravenous injection of 5 cc. of tumor filtrate. When the birds were examined at a later date, they all showed tumor formation in the breast muscles in the region of the tar injections (Fig. 2). Evidently tar resembles Scharlach R in its localizing effect.

Experiments with Kieselguhr.

The reaction produced by kieselguhr in the tissues is a peculiar one, and has been studied by Podwyssozki (7) and others. It consists very largely of giant cells, probably of the foreign body type, with little of the ordinary connective tissue (Fig. 3). It seemed of interest to attempt localization with this substance. Accordingly five hens were inoculated with a mixture of kieselguhr in Ringer's solution, 1 cc. being injected into each breast. This was repeated in a week, and then 7 days after the second injection, the tumor filtrate was passed into a wing vein. One of the birds died early in the experiment and was discarded. Of the remainder, only one out of the four developed a tumor of the breast muscle. All showed tumor localizations in other parts of the body.

This experiment was carried out under conditions very similar to those of the second experiment with Scharlach R and the one with tar. The same filtrate was used for the three. The differing results would suggest that the nature of the tissue reaction is of importance in determining the degree of localization.

Experiments with Embryonic Tissue.

In these experiments the local change was induced with fresh chick embryo tissue.

First Experiment.—7 to 10 day embryos were hashed up in Ringer's solution, and 1 cc. injected into the breast muscle of each of five hens. Only one injection of embryo tissue was given to each bird. When, after 10 days, well formed embryomas had appeared, an injection of 5 cc. of fresh tumor filtrate was given into a wing vein. After 21 days had elapsed, a time presumably sufficient for localiza-

tion to have taken place in or around the embryo nodules, the birds were killed and examined. Of the five used, only one, showed such a localization of the growth. That the filtrate was active and the birds not resistant was shown by the fact that all five developed tumors in other regions. Furthermore the filtrate was the same as that used for the second Scharlach R experiment where localization took place in the areas of induced reaction in all the fowls injected.

In view of the results obtained in the second Scharlach R experiment, it was decided to reduce the time between the injection of embryo tissue and the injection of the filtrate.

Second Experiment.—Four hens were injected into the pectoral muscles with 1 cc. of tissue. Two of these were then injected intravenously with 5 cc. of tumor filtrate 3 days after receiving the embryo tissue, and the other two received their injections 5 days after the embryo hash. The results were as follows:

(*a*) The chickens injected with filtrate 3 days after the introduction of the embryonic tissues had numerous tumor nodules in and around the embryoma (Fig. 4), all being in contact with the embryonic tissue. These findings were verified by microscopic examination.

(*b*) The chickens which received the filtrate 5 days after the embryonic tissue injection showed macroscopically no evidence of tumor localization but microscopic examination disclosed in one fowl a few scattered areas of sarcomatous transformation in the embryonic tissue.

The same filtrate was used in both of these groups and its potency was shown by the fact that all of the fowls developed tumors in other parts of the body.

While the first and second experiments were carried out with different filtrates it seems justifiable to compare the results, since in both the filtrate was very active as shown by the fact that all of the fowls developed tumors elsewhere than in the breast. The findings suggest that the reaction induced by the injection of embryo tissue is only active as a localizing factor in its early stages.

DISCUSSION.

Localization of the agent of Chicken Tumor 1, resulting in tumor formation has been accomplished by inducing the development of reaction tissue in the pectoral muscles prior to the intravenous injections of fresh tumor filtrate.

The irritants successfully used for the purpose differ somewhat in their effect, those in one group, Scharlach R, tar and embryo tissue,

eliciting an ordinary subacute reaction of the fibroblastic type, while kieselguhr gives rise to the development of a peculiar tissue consisting largely of giant cells. In the experiment with irritants of the first type, the tumor localization occurred most frequently in the early stages of the reaction when the reaction cells were young and actively growing. As the reaction tissue became older less localization in it took place.

While the number of fowls injected with kieselguhr was relatively few, the small proportion of positive results obtained with this substance has some significance, since the filtrate employed was the same which caused tumors in all of the fowls that had been injected with Scharlach R and tar. It may be concluded tentatively that the peculiar reaction tissue elicited by kieselguhr is less favorable to the localization of the tumor-producing agent than is that induced by other substances. It is of interest to note that kieselguhr is less active in localizing vaccine virus after intravenous injection than is the case with a variety of other substances (10).

Earlier work by Murphy and Rous (11) has shown that in order to produce Chicken Tumor 1 in the embryo the agent must be brought into contact with mesodermal tissue. It is reasonable to suppose that young and actively growing connective tissue will be more susceptible than older tissues to the influence of the tumor agent. This supposition is supported by the above experiments. They would also suggest that it is the reaction to injury rather than the injury as such which renders a tissue susceptible to the tumor-producing activity of the agent.

SUMMARY.

A localization out of the blood stream of the agent causing a chicken tumor, with the subsequent development of the growth can be brought about in the breast muscle, by inducing in this tissue a reaction by such substances as Scharlach R, tar, embryonic tissue and kieselguhr. Localization in the reaction tissue elicited by kieselguhr takes place relatively infrequently. The earlier stages of the reaction induced by these substances localize the tumor agent more regularly than the later stages of the reaction.

BIBLIOGRAPHY.

1. Kiedel, A., and Zimmermann, E. L., *Am. J. Syph.*, 1918, ii, 83.
2. Chesney, A. M., and Kemp, J. E., *J. Exp. Med.*, 1925, xli, 487.
3. Gye, W. E., and Kettle, E. H., *Brit. J. Exp. Path.*, 1922, iii, 241.
4. Levaditi, C., and Nicolau, S., *Compt. rend. Soc. biol.*, 1922, lxxxvi, 986.
5. Lusena, M., *Sperimentale*, 1925, lxxix, 969; *Cancer Rev.*, 1926, i, 12.
6. Rous, P., Murphy, Jas. B., and Tytler, W. H., *J. Am. Med. Assn.*, 1912, lviii, 1751.
7. Podwyssozki, W., *Beitr. path. Anat. u. allg. Path.*, 1910, xlvii, 270.
8. Jones, F. S., and Rous, P., *J. Exp. Med.*, 1914, xx, 404.
9. Rous, P., Robertson, O. H., and Oliver, J., *J. Exp. Med.*, 1919, xxix, 305.
10. Reynals, F. D., *J. Exp. Med.*, 1928, xlvii, 389.
11. Murphy, Jas. B., and Rous, P., *J. Exp. Med.*, 1912, xv, 119.

EXPLANATION OF PLATES.

PLATE 19.

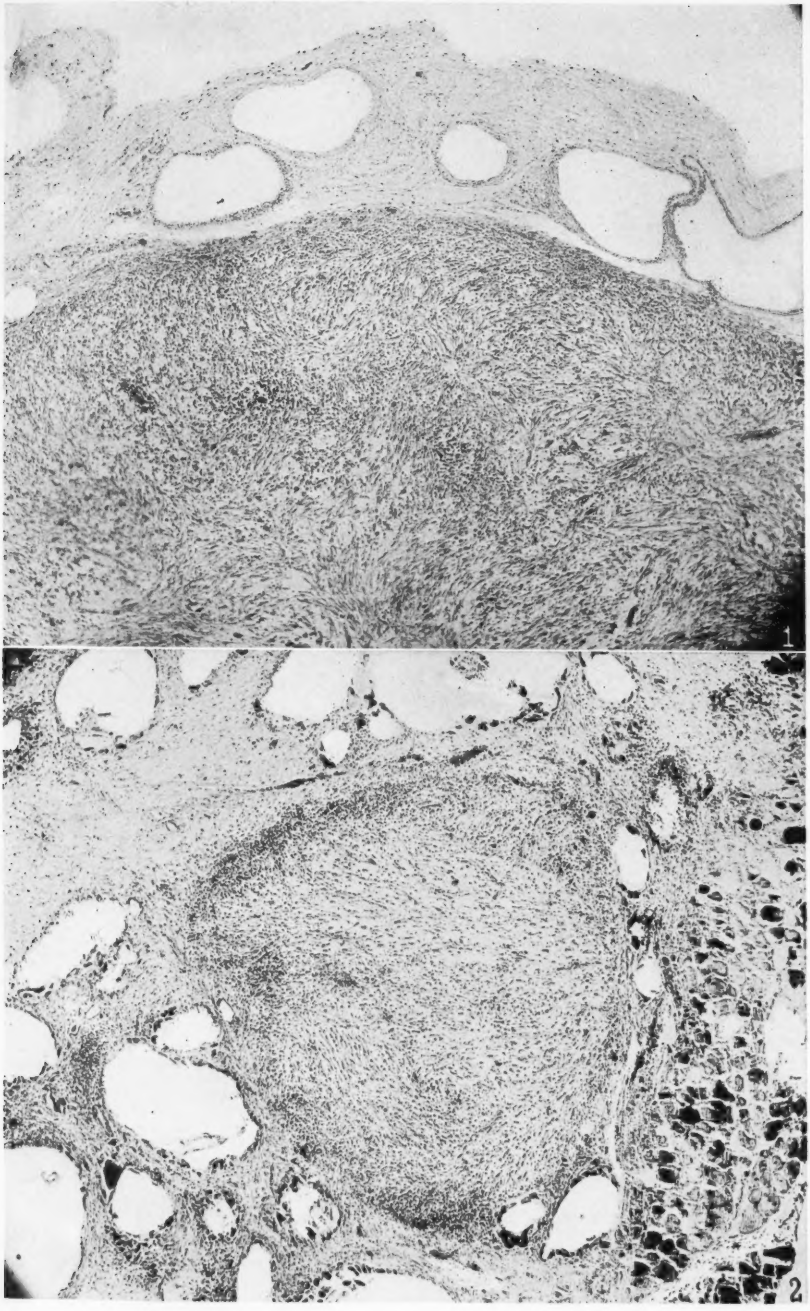
FIG. 1. Localization of Chicken Tumor 1 in a region injected with Scharlach R in olive oil. Clear spaces which contained the Scharlach R are surrounded by young connective tissue, and lying alongside is a mass of tumor tissue.

FIG. 2. Localization of chicken tumor in a region injected with tar. Clear spaces indicate position of tar, and in between these is much young connective tissue. In the center is the tumor nodule. The dark spots are more or less damaged muscle fibers.

PLATE 20.

FIG. 3. Area of kieselguhr reaction in chicken muscle. This is composed mainly of giant cells with very little connective tissue. There is no localization of the tumor agent.

FIG. 4. Localization in a region of embryoma formation. A well formed tumor nodule is seen above, and below is a mixture of embryonic tissues, composing the embryoma.



(Mackenzie and Sturm: Localization of chicken tumor agent.)

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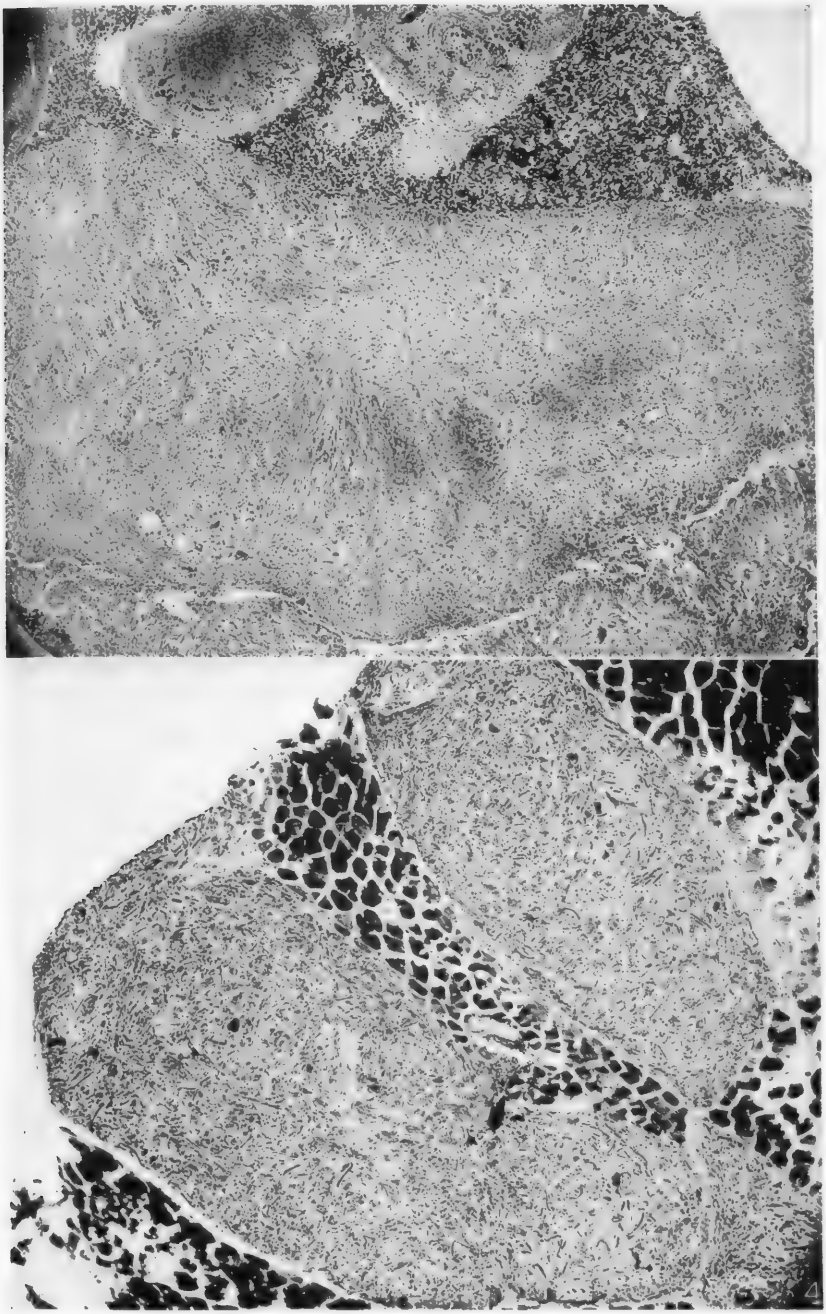

(Mackenzie and Sturm: Localization of chicken tumor agent.)

THE EFFECT OF DIGESTS OF PURE PROTEINS ON CELL PROLIFERATION.

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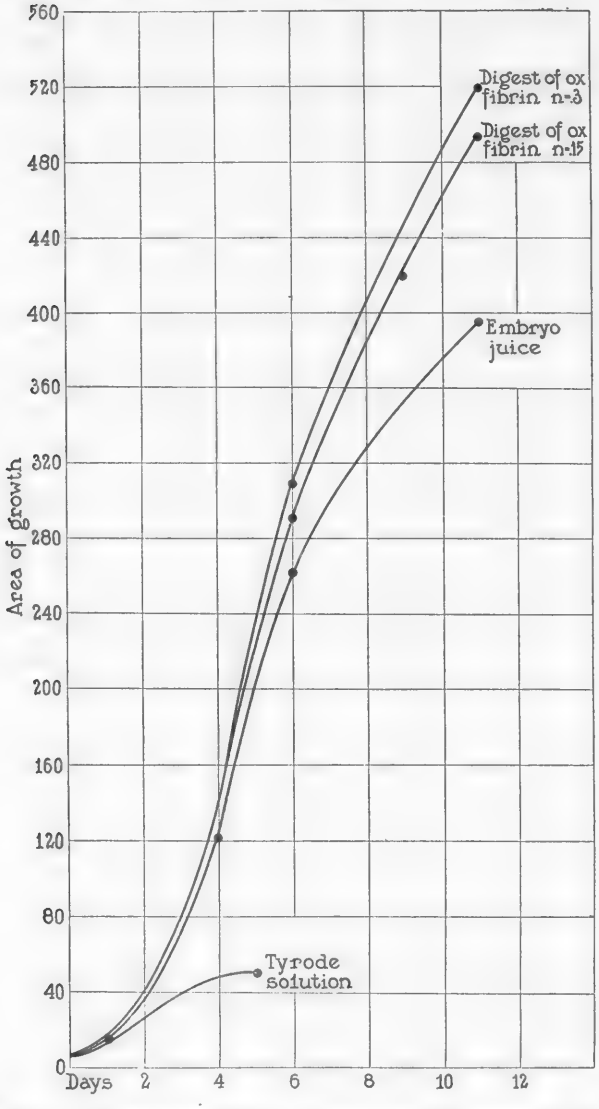
It has been reported that fibroblasts, epithelial cells, and leucocytes display great proliferative activity when cultivated in the peptic digestion products of proteins.[1] That statement was based on experiments with digests of complex substances, such as embryo pulp, egg white, commercial ox fibrin, and Witte's peptone. In order to ascertain conclusively whether cell multiplication was due to the hydrolytic products of the proteins themselves, or to other substances in the material used, the experiments were repeated with proteins which were purified before being hydrolyzed. For this purpose, fibrin, egg albumin, and edestin were used, because the digestion products of commercial fibrin have been found to possess a greater growth-promoting effect than any other substance, and because egg albumin and edestin may be purified by crystallization.

Effect of Split Products of Fibrin on Cell Proliferation.

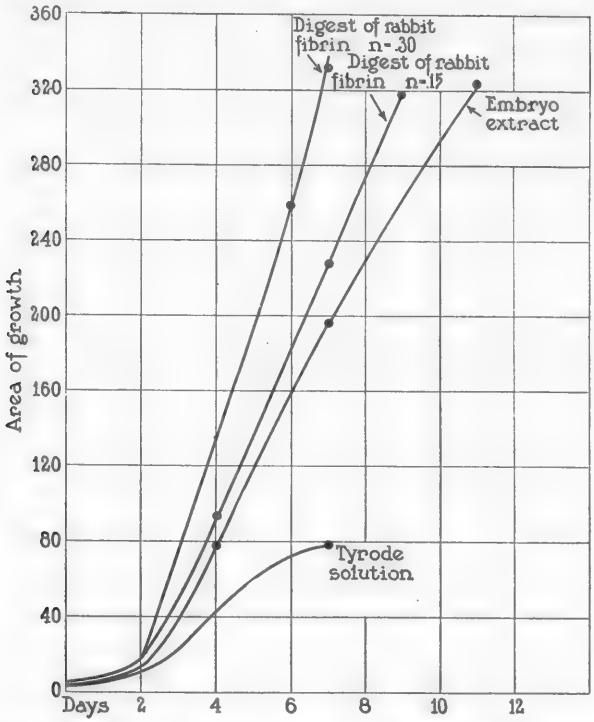
The fibrin was prepared from both ox and rabbit blood. The ox blood was citrated by drawing it into a 2.5 per cent sodium citrate solution in 0.9 per cent salt solution, using 100 cc. per liter of blood. The rabbit blood was taken in paraffined tubes and kept in ice to prevent coagulation. After centrifugation of the blood, the upper layers only were drawn off and again centrifuged in order to eliminate all the corpuscles from the plasma. The fibrin was precipitated by half saturation with sodium chloride and washed three times with a half saturated salt solution, three times with 0.9 per cent sodium chloride, and then with large volumes of water. The dry fibrin was digested for 24 hours at 40°C., 2.5 gm. of fibrin being suspended in 40 cc. of 0.5 per cent pepsin in N/20 HCl. Since some fibrin remained undigested, a large proportion of the primary split products was obtained in this way. The solutions were neutralized with N/1 NaOH,

[1] Carrel, A., and Baker, L. E., *J. Exp. Med.*, 1926, xliv, 503.



TEXT-FIG. 1. Experiment 9162 D. Comparison of the growth of fibroblasts from fresh embryo heart in a digest of purified ox fibrin, with that in embryo juice and Tyrode solution.

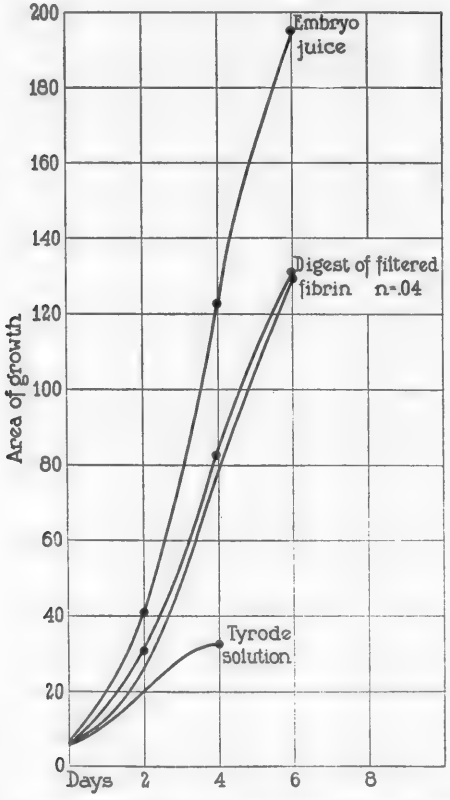
boiled to one-half volume in order to remove the coagulable substances and expel the toluene which was used as a preservative. After determination of the cryoscopic point, the solution was brought to isotonicity and a pH of 7.4. These digests were tested at various concentrations on fibroblasts from embryonic



TEXT-FIG. 2. Experiment 3606 A. Comparison of the growth of fibroblasts from fresh embryo heart in a digest of purified rabbit fibrin, with that in embryo juice and Tyrode solution.

heart cultivated in flasks according to the usual method, the fluid being placed on the surface of a coagulum of diluted plasma. Every 48 hours, the fluid was removed, the culture washed with Tyrode solution, and fresh fluid supplied. The area of the colony was traced under a projectoscope and measured with a planimeter.

The digest of purified ox fibrin, and also that of rabbit fibrin, at an N concentration of from 0.15 to 0.3, caused a large growth of fibroblasts from embryonic heart, as large or larger than that which occurred in



TEXT-FIG. 3. Experiment 9225 D. Comparison of the growth of fibroblasts from fresh embryo heart in a digest of ox fibrin from filtered plasma, with that in embryo juice and Tyrode solution. The concentration of nitrogen in this digest was much less than in the previous ones.

embryo juice (Text-figs. 1, 2; Table I). The results were entirely comparable with those obtained when commercial fibrin was used. It is evident, therefore, that the substances which cause this rapid

proliferation of cells are the hydrolytic products of fibrin and not the impurities contained in commercial fibrin such as blood corpuscles. One sample of ox plasma was filtered through a Berkefeld filter. As the filter soon became clogged, only a small amount of fibrin was obtained. The quantity was so small that only a dilute solution of the digest could be used in the culture medium at a nitrogen concentration of 0.04 per cent. Nevertheless, a considerable growth of fibroblasts occurred (Text-fig. 3).

Effect of Crystalline Egg Albumin Digests on Cell Proliferation.

The egg albumin was purified by three crystallizations with ammonium sulfate. To remove the ammonium sulfate which previous experience had shown to be toxic even after long dialysis of the albumin, two methods were used. In the first, the crystals were washed three times for 24 hours with a solution of saturated sodium chloride containing 1 per cent acetic acid. This denatured part of the albumin. The second method consisted of recrystallization three times with a mixture of sodium and potassium sulfates, making a total of six crystallizations. The albumin was finally dialyzed in very permeable collodion bags for 7 days in running tap water and 3 days in distilled water.

Several digests were made of this albumin, the quantity of pepsin, the time of digestion, and the concentration of the solution being varied. Some crystalline albumin was coagulated by heat before digestion and the process interrupted as soon as the albumin was transformed into soluble material. A large proportion of primary split products was thus secured. The methods of preparing the digests are summarized in Table II. In all cases, the solutions were boiled and prepared in the same manner as the fibrin digests. These digests proved toxic at nitrogen concentrations as high as those used for the fibrin digests and therefore were diluted with Tyrode solution to a nitrogen concentration of 0.17 to 0.07 per cent before being used as a culture medium. They were tested in the manner outlined above on fibroblasts growing from embryonic heart. The increased area of growth of fibroblasts from fresh heart in the albumin digests over that of control tissues in Tyrode solution varied from 75 to 400 per cent, according to the digest used (Table III). Some of the best results were obtained with digests prepared from heat denatured albumin. In order to ascertain whether this previous denaturation influenced

the nutritive value of the digests, two preparations were made (x–512 and x–513) from the same solution, under identical conditions except that one (x–512) was first denatured by heating in N/100 HCl solution for 15 minutes at 60°C. No differences were found in their nutritive effect.

The digests of crystalline albumin did not, however, promote as large or rapid a growth of fibroblasts as is caused by the digests of fibrin or by embryo juice. The area of growth was about equal to, but not as dense as that in embryo juice diluted to 50 per cent of its usual concentration (Table IV). Moreover, the cells of the controls in the diluted embryo juice remained in normal condition, while those in the digests finally degenerated, an evidence that although the albumin split products cause growth, they are incomplete from the nutritive standpoint.

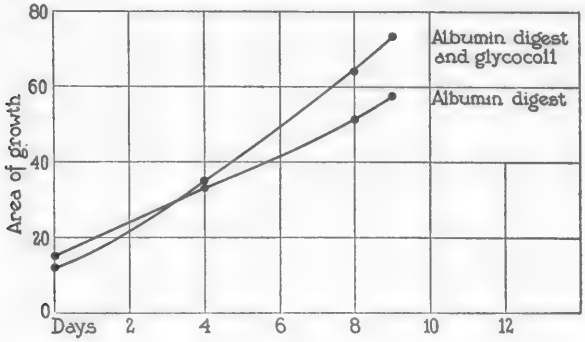
The deficiency of the egg albumin digests was still more evident when a 15 year old strain of chicken fibroblasts was used in the experiments. The growth in the albumin digests was very much smaller than that of the controls in embryo juice, and only slightly greater than that of the controls in Tyrode solution. In all cases, the growth ceased after a few days, the cells became filled with fat, and the plasma clot usually liquefied immediately around the tissue, making it impossible to secure accurate quantitative data. It is probable that the larger growth of the fibroblasts from embryonic heart is due to the presence in the fresh tissue of some substances which are needed to supplement the nutritive action of the digestion products of crystalline egg albumin.

Effects on Cell Proliferation of Digests of Crystalline Egg Albumin and Casein Supplemented by Glycocoll and Nucleic Acid.

It seemed probable that the great differences in the nutritive value of the digests of fibrin and of crystalline egg albumin might be due to the difference in their amino acid content. Since crystalline albumin contains no glycocoll, the addition of sufficient glycocoll[2] solution to bring its concentration up to that of the fibrin digests was tried, and

[2] The glycocoll solution contained 50 mg. of crystalline glycocoll in 100 cc. of Tyrode solution. It was sterilized by filtration through a Berkefeld filter.

also the addition of a digest of gelatin,[3] a protein particularly rich in glycocoll. For these experiments, a pure strain of sarcomatous fibroblasts of the rat was used, since it was the only pure strain of fibroblasts immediately available which did not cause liquefaction of the coagulated plasma. These cells grew very slightly in the digest of crystalline albumin, in fact, hardly any more than when cultivated in Tyrode solution. The addition of glycocoll increased the rate of growth about 70 per cent (Text-fig. 4, Table V), and the gelatin digest about 61 per cent. Similar results were obtained when the experi-



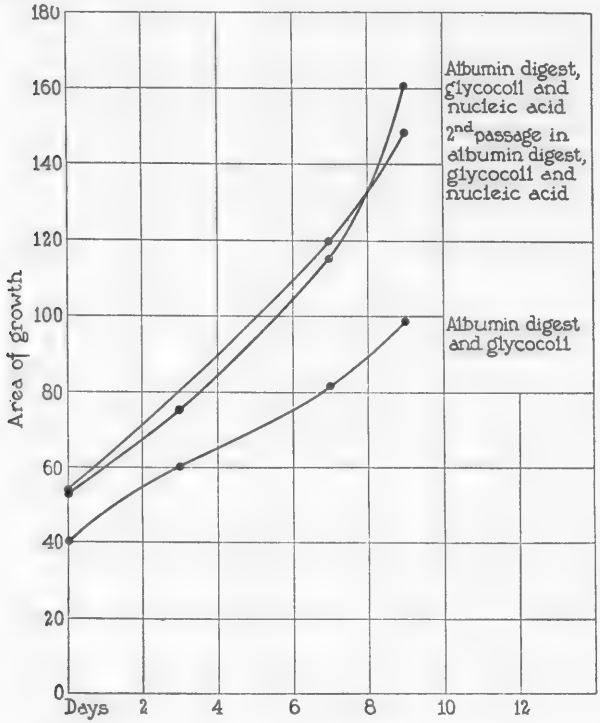
TEXT-FIG. 4. Experiment 6140 C. Effect of the addition of glycocoll to a digest of crystalline egg albumin on the rate of growth of sarcomatous fibroblasts of the rat.

ments were repeated with digests of casein[4] which contains only an exceedingly small quantity of glycocoll (Table VI), showing beyond doubt that glycocoll is an essential component of the culture medium,

[3] The gelatin digest was prepared by incubating 5 gm. of gelatin with 1 gm. of pepsin in 200 cc. of N/20 HCl at 37°C. for 24 hours. It was neutralized, boiled, and adjusted for cultures as usual. It contained 0.45 per cent of nitrogen and 0.048 per cent of amino nitrogen. Before use on the cultures, it was diluted to 0.25 per cent of nitrogen.

[4] The casein digest was prepared by incubating 10 gm. of casein at 37°C. for 24 hours, with 0.5 per cent of pepsin in 200 cc. of N/20 HCl. This was also neutralized, boiled, made isotonic, etc. It was diluted to 0.4 per cent of nitrogen before being used on the cultures. Its ratio of total to amino nitrogen was 7.2, and 67 per cent of its nitrogen was in the form of proteose.

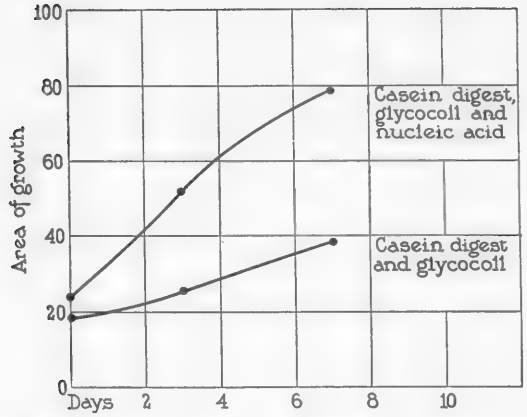
either as such, or in peptide form, and that one of the reasons for the different nutritive actions of protein digests lies in their varying amino acid content.



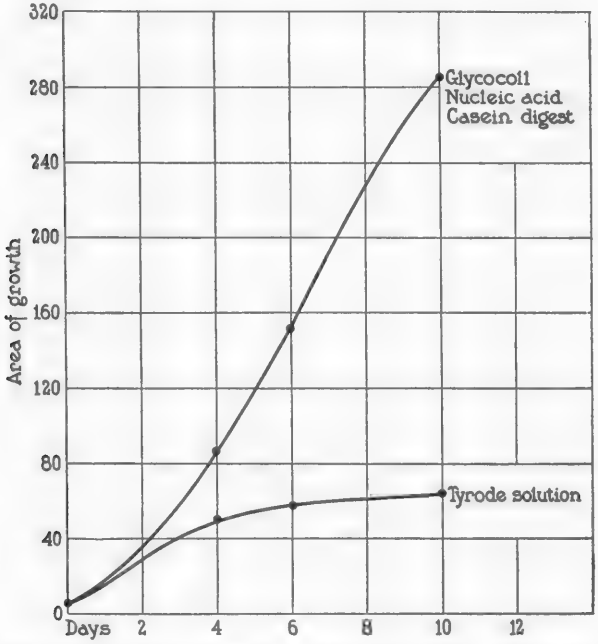
TEXT-FIG. 5. Experiment 6162 C. Effect of the addition of nucleic acid to a mixture of glycocoll and a digest of crystalline egg albumin on the rate of growth of sarcomatous fibroblasts of the rat. The tissue having the nucleic acid in its medium grew uniformly through two passages, while the control in glycocoll and albumin digest died at the end of the 1st passage.

However, even after the addition of glycocoll, the growth was small and the tissues survived only a few days longer than in Tyrode solution. Then, thymus nucleic acid[5] was added to the medium. This

[5] The thymus nucleic acid solution contained 0.1 gm. of nucleic acid in 100 cc. of Ringer's solution. N/1 NaOH was added to dissolve it and bring the pH to 7.4. It was sterilized by filtration through a Berkefeld filter.



TEXT-FIG. 6. Experiment 9454 D. Effect of the addition of nucleic acid to a mixture of glycocoll and a digest of casein, on the rate of growth of sarcomatous fibroblasts of the rat.



TEXT-FIG. 7. Experiment 9504 D. Growth of a 15 year old strain of normal chicken fibroblasts in a mixture of glycocoll, casein digest, and nucleic acid.

greatly improved its nutritive value (Text-fig. 5). An additional increase of approximately 97 per cent in the area of growth was obtained when nucleic acid was added to a mixture of crystalline egg albumin digest and glycocoll (Table VII), and an additional increase of approximately 193 per cent when it was added to a mixture of glycocoll and casein digest (Table VIII). In fact, the strain of malignant fibroblasts grows at a rapid rate in the very simple mixture of just three substances: digest of casein or crystalline egg albumin, glycocoll, and thymus nucleic acid dissolved in Tyrode solution. Growth in a mixture of the three substances was better than in any two of them (Tables IX and X). Not only did the presence of nucleic acid increase the rate of growth, but it also greatly lengthened the time of survival of the tissues. In the digest of crystalline egg albumin, glycocoll, and nucleic acid, colonies of the sarcomatous fibroblasts have grown actively for more than 20 days. In the mixture of glycocoll, nucleic acid, and casein digest, some of the cultures were still active after more than a month. The colonies have been divided several times, showing that a considerable increase in the mass of the tissues has occurred. Still more encouraging is the fact that the cells remained in normal condition throughout the experiment, although the rate of growth was much less than that of colonies cultivated in embryo juice. Many other substances have been added to this simple medium, such as iron, vitamines, salts, cystine, cholesterol, etc., but as yet no conclusive evidence of their nutritive value has been obtained.

Effect of Vegetable Protein Digests on Cell Multiplication.

Since both crystalline egg albumin and casein, which have been used in the synthetic media, are deficient in glycocoll experiments were undertaken with digests of edestin which is rich in glycocoll and can also be crystallized. Crystalline edestin obtained from Hoffman-La Roche was used. Digests of this protein[6] were found to promote the proliferation of rat sarcomatous fibroblasts much more actively than either those of casein or crystalline egg albumin. It is

[6] The edestin digest was prepared by digesting 2.5 gm. of crystalline edestin in 100 cc. of 0.5 per cent pepsin in N/20 HCl for 20 hours. It was also neutralized, boiled, and made isotonic.

TABLE I.

Comparison of the Rate of Growth of Fibroblasts from Embryo Heart in Embryo Juice and in a Digest of Purified Fibrin.

Experiment No.	Preparation No.	Nitrogen concentration	Time of growth	Area[7] of growth in embryo juice. Control	Area[7] of growth in fibrin digest. Experiment	Ratio: area of experiment to area of control	Remarks
		per cent	*days*				
6068 C 1–2	x-525	0.208	8	87	83	0.95	Ox fibrin
6017 C 1–3	x-525	0.208	7	72	126	1.75	" "
3606 A 1	x-468	0.30	7	200	356	1.78	Rabbit "
3606 A 2	x-468	0.15	7	200	240	1.20	" "
3606 A 3	x-468	0.30	7	200	194	0.97	" "
3606 A 4	x-468	0.15	7	200	170	0.85	" "
9162 D 1	x-467	0.15	10	370	460	1.24	Ox "
9162 D 2	x-467	0.30	10	370	480	1.30	" "
Average.....						1.26	

TABLE II.

Methods of Preparing Albumin Digests.

Preparation No.	Kind of albumin	Preliminary treatment	Volume of solution used	Pepsin	Concentration of HCl	Time of digestion	Ratio: total to amino nitrogen
			cc.	*per cent*		*hrs.*	
x-360	Crystallized 3 times. Washed, 1 per cent acetic in saturated NaCl.	Coagulated by heat	200	0.5	N/20	5	4.7
x-383	" "	Acid denatured	60	0.5	N/30	4	7.1
x-502	" "	" "	50	0.5	N/20	5	8.2
x-503	" "		72	0.5	N/20	10	6.5
x-504	" "	Denatured by heat	72	0.5	N/20	5	7.1
x-512	Crystallized 6 times, 3 from Na_2SO_4 and 3 from K_2SO_4	" " "	80	0.5	N/20	15	6.5
x-513	" "	Not denatured	80	0.5	N/20	15	6.4
x-534[8]	" "	Coagulated by heat	200	0.25	N/20	7	6.7

[7] This is the area in square centimeters of the projected image of the colony of cells, and is 62 times the size of the colony.

[8] 72 per cent of the nitrogen of this preparation was precipitated by saturated sodium sulfate at 37°C. Therefore, it consisted largely of proteose.

TABLE III.

Effect of Digests of Crystalline Egg Albumin on the Rate of Growth of Fibroblasts from Embryonic Heart. Tyrode Solution Used as Control.

Experiment No.	Preparation No.	Ratio: total N to amino N	Nitrogen concentration	Time of growth	Area[7] of growth in Tyrode solution. Control	Area[7] of growth in albumin digest. Experiment	Ratio: increased area of experiment to control
				days			
8816 D	x-360	4.7	0.085	7	50	210	4.2
3922 C	x-360	4.7	0.085	8	90	170	1.9
3922 C	x-360	4.7	0.170	9	110	353	3.2
3353 A	x-360	4.7	0.085	8	110	170	1.5
3353 A	x-360	4.7	0.170	8	110	270	2.5
3961 C	x-360	4.7	0.170	10	70	140	2.0
3968 C	x-360	4.7	0.170	4	38	68	1.8
8818 D	x-360	4.7	0.170	5	49	95	1.9
3347 A	x-360	4.7	0.170	5	48	98	2.0
3991 C	x-383	7.1	0.120	7	55	268	4.9
9240 D	x-502	8.2	0.070	6	50	88	1.8
9240 D	x-503	6.5	0.070	6	50	95	1.9
9240 D	x-504	7.1	0.070	6	50	95	1.9
4855 C	x-513	6.4	0.120	9	10	47	4.7
4855 C	x-512	6.5	0.120	9	10	70	7.0
4909 C	x-512	6.5	0.120	7	15	61	4.0
4909 C	x-513	6.4	0.120	7	15	60	4.0
4828 C	x-512	6.5	0.120	9	10	46	4.6
4828 C	x-513	6.4	0.120	9	10	38	3.8
Average.....							3.1

TABLE IV.

Comparison of the Effect of Digests of Crystalline Egg Albumin and of Embryo Extract Diluted 50 Per Cent on the Rate of Growth of Fibroblasts from Fresh Embryo Heart.

Experiment No.	Preparation No.	Nitrogen concentration	Time of growth	Area[7] of growth in embryo extract. Control	Area[7] of growth in albumin digest. Experiment	Ratio: area of experiment to area of control	Remarks
		per cent	*days*				
4855 C	x-512	0.12	9	57	70	1.23	Thin growth in experiments
4828 C	x-512	0.12	9	45	46	1.02	" " " "
4909 C	x-512	0.12	7	52	61	1.17	" " " "
4854 C	x-512	0.12	10	28	32	1.14	" " " "
4855 C	x-513	0.12	9	57	47	0.82	" " " "
4828 C	x-513	0.12	9	45	38	0.84	" " " "
4909 C	x-513	0.12	7	52	60	1.15	" " " "
4854 C	x-513	0.12	10	28	29	1.03	" " " "
Average.....						1.05	

TABLE V.

Effect of the Addition of Glycocoll to a Digest of Crystalline Egg Albumin[8] on the Rate of Growth of Sarcomatous Fibroblasts of the Rat.

Experiment No.	Preparation No.	Nitrogen concentration	Time of growth	Area[7] of growth. Control[9]	Area[7] of growth. Experiment[10]	Ratio: area of experiment to area of control
		per cent	*days*			
6171 C	x-534	0.13	9	84	103	1.23
6172 C	x-534	0.13	9	42	61	1.45
6142 C	x-534	0.13	9	27	36	1.33
6140 C	x-534	0.13	9	24	53	2.20
6092 C	x-534	0.13	7	18	47	2.61
Average..						1.76

TABLE VI.

Effect of the Addition of Gelatin Digest to Casein Digest on the Growth of Sarcomatous Fibroblasts of the Rat.

Experiment No.	Preparation No.	Nitrogen concentration	Time of growth	Area[7] of growth. Control[11]	Area[7] of growth. Experiment[12]	Ratio: area of experiment to area of control
		per cent	*days*			
455 H	x-542 x-545	0.323	4	16	35	2.18
476 H	x-542 x-545	0.323	9	31	44	1.42
480 H	x-542 x-545	0.323	7	18	28	1.56
481 H	x-542 x-545	0.323	7	17	22	1.29
Average..						1.61

[9] The control medium consisted of 1 cc. of albumin digest and 2 cc. of Tyrode solution.

[10] The experimental medium consisted of 1 cc. of albumin digest, 1 cc. of Tyrode solution, and 1 cc. of glycocoll solution.

[11] The control medium consisted of 1 cc. of casein digest and 1 cc. of Tyrode solution.

[12] The experimental medium consisted of 1 cc. of casein digest and 1 cc. of gelatin digest.

TABLE VII.

Effect of the Addition of Nucleic Acid to a Mixture of Crystalline Egg Albumin Digest[8] *and Glycocoll on the Rate of Growth of Sarcomatous Fibroblasts of the Rat.*

Experiment No.	Preparation No.	Nitrogen concentration	Time of growth	Area[7] of growth. Control[13]	Area[7] of growth. Experiment[14]	Ratio: area of experiment to area of control	Remarks
		per cent	*days*				
6162 C	x-534 x-551 x-554	0.1	9	58	108	1.87	1st passage
6162 C	x-534 x-551 x-554	0.1	9	No growth	104		2nd "
6163 C	x-534 x-551 x-554	0.1	9	53	110	2.07	
Average.....						1.97	

TABLE VIII.

Effect of the Addition of Nucleic Acid to a Mixture of Casein Digest and Glycocoll on the Rate of Growth of Sarcomatous Fibroblasts of the Rat.

Experiment No.	Preparation No.	Nitrogen concentration	Time of growth	Area[7] of growth. Control[15]	Area[7] of growth. Experiment[16]	Ratio: area of experiment to area of control
		per cent	*days*			
9488 D	x-539 x-551 x-554	0.14	8	14	31	2.22
9492 D	x-539 x-551 x-554	0.14	8	15	48	3.20
9441 D	x-539 x-551 x-554	0.14	6	40	37	0.92
9454 D	x-539 x-551 x-554	0.14	8	10	54	5.40
Average.....						2.93

[13] The control medium consisted of 1 cc. of albumin digest, 1 cc. of glycocoll solution, and 1 cc. of Tyrode solution.

[14] The experimental medium consisted of 1 cc. of albumin digest, 1 cc. of glycocoll solution, and 1 cc. of nucleic acid solution.

[15] The control medium consisted of 1 cc. of casein digest, 1 cc. of glycocoll solution, and 1 cc. of Tyrode solution.

[16] The experimental medium consisted of 1 cc. of casein digest, 1 cc. of glycocoll solution, and 1 cc. of nucleic acid solution.

TABLE IX.

Effect of the Addition of Casein Digest to a Mixture of Nucleic Acid and Glycocoll on the Rate of Growth of Sarcomatous Fibroblasts of the Rat.

Experiment No.	Preparation No.	Nitrogen concentration	Time of growth	Area[7] of growth. Control[17]	Area[7] of growth. Experiment[18]	Ratio: area of experiment to area of control
		per cent	*days*			
6196 C	x-539 x-551 x-554	0.14	9	34	74	2.18
6219 C	x-539 x-551 x-554	0.14	9	46	78	1.70
Average						1.94

TABLE X.

Effect of the Addition of Glycocoll to a Mixture of Casein Digest and Nucleic Acid on the Rate of Growth of Sarcomatous Fibroblasts of the Rat.

Experiment No.	Preparation No.	Nitrogen concentration	Time of growth	Area[7] of growth. Control[19]	Area[7] of growth. Experiment[20]	Ratio: area of experiment to area of control
		per cent	*days*			
9463 D	x-539 x-551 x-554	0.14	9	29	44	1.52
9472 D	x-539 x-551 x-554	0.14	7	28	39	1.39
9443 D	x-539 x-551 x-554	0.14	8	41	73	1.78
Average						1.56

[17] The control medium consisted of 1 cc. of nucleic acid solution, 1 cc. of glycocoll solution, and 1 cc. of Tyrode solution.

[18] The experimental medium consisted of 1 cc. of nucleic acid solution, 1 cc. of glycocoll solution, and 1 cc. of casein digest.

[19] The control medium consisted of 1 cc. of casein digest, 1 cc. of nucleic acid solution, and 1 cc. of Tyrode solution.

[20] The experimental medium consisted of 1 cc. of casein digest, 1 cc. of nucleic acid solution, and 1 cc. of glycocoll solution.

TABLE XI.

Effect of a Mixture of Protein Digest, Glycocoll, and Nucleic Acid on the Growth of Normal Fibroblasts. Tyrode Solution Used as Control.

Experiment No.	Preparation No.	Nitrogen concentration	Time of growth	Area of growth. Control	Area of growth. Experiment	Ratio: area of experiment to area of control	Remarks
		per cent	*days*				
3847 A	x-539, x-551, x-554	0.14	5	66.5	95.4	1.43	Pure strain of chicken fibroblasts; casein digest
3830 A	x-534, x-551, x-554	0.10	6	28	56	2.00	Pure strain of chicken fibroblasts; crystalline egg albumin digest
3917 A	x-551, x-608, x-653	0.06	7	40	98	2.45	Pure strain of rat fibroblasts; crystalline egg albumin digest
3881 A	x-539, x-551, x-554	0.14	5	28	42	1.50	Pure strain of chicken fibroblasts; casein digest
9504 D	x-539, x-551, x-554	0.14	10	64	280	4.38	Pure strain of chicken fibroblasts; casein digest
144 G	x-551, x-554, x-562	0.14	6	14	42	3.00	Pure strain of chicken fibroblasts; casein digest
6298 C	x-539, x-551, x-554	0.14	5	70	135	1.93	Fibroblasts from chicken embryo heart; casein digest
6298 C	x-539, x-551, x-554	0.14	5	105	205	1.95	Fibroblasts from chicken embryo heart; casein digest
Average.....						2.33	

interesting to note that the digestion products of a vegetable protein, as well as those of animal origin, can be used by these cells. This was also found to be true of the gluten of wheat. In addition, normal chicken fibroblasts also proliferate in digests of both of these proteins.

Effects of Artificial Media on Normal Tissues.

Although the effect of adding glycocoll and nucleic acid to digests of proteins was tested on a strain of sarcomatous fibroblasts of the rat, it was also demonstrated that a pure strain of normal chicken fibroblasts will grow for a limited period in a mixture of protein digest, nucleic acid, and glycocoll. Text-fig. 7 gives the curve of growth for a strain of normal fibroblasts in casein digest, nucleic acid, and glycocoll. Similar results have been obtained with crystalline egg albumin digest, glycocoll, and nucleic acid. Although the rate of growth of normal fibroblasts is much larger in this artificial medium than in Tyrode solution (Table XI), the cells accumulate dark granulations in their cytoplasm, and die after 8 or 10 days. Liquefaction of the plasma clot often occurs. These cells seem to be more sensitive to the deficiencies of the artificial media than do the sarcomatous fibroblasts of the rat.

CONCLUSIONS.

1. The pepsin hydrolytic products of the pure proteins, crystalline egg albumin, crystalline edestin, and purified fibrin, are utilized by fibroblasts for their proliferation. It appears, therefore, that the growth of fibroblasts in fibrin digests and in the proteose of Witte's peptone is due to the split products of the protein itself and not to accompanying cellular constituents or other impurities. Preliminary denaturation of the protein, as carried out in these experiments, does not alter the nutritive properties of the digest.

2. The digests of the pure proteins employed are deficient in certain substances and do not meet the entire nutritive requirement of the cells for an unlimited period of time. Some supplementary nutritive substances are present in fresh embryonic heart tissue. This circumstance explains the growth of fibroblasts from fresh embryonic heart in digests which do not promote the growth of a pure strain of fibroblasts.

3. Glycocoll and nucleic acid have been found to supplement the nutritive action of pure protein digests for sarcomatous fibroblasts, and to increase greatly the length of life of the tissues.

4. Vegetable proteins, as well as animal proteins, yield proteolytic products which promote the multiplication of fibroblasts.

EFFECT OF LIVER AND PITUITARY DIGESTS ON THE PROLIFERATION OF SARCOMATOUS FIBROBLASTS OF THE RAT.

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Since the experiments reported in the preceding paper[1] showed that the hydrolytic products of pure proteins do not support the indefinite proliferation of fibroblasts, and that they need to be supplemented by some substance present in fresh tissues, it seemed probable that a more complete nutritive medium might be obtained from the digests of certain tissues or glands instead of from pure proteins. The purpose of this paper is to describe the effects of the peptic digestion products of calf liver and the anterior lobe of the pituitary gland on the growth, not of fresh tissues, but of a pure strain of sarcomatous cells after several months of life *in vitro*.

Preparation of the Digests.

The liver digest was prepared by incubating 16 gm. of strictly fresh, finely ground liver for 20 hours at 37°C. in 200 cc. N/20 HCl, containing 1 gm. of Fairchild's pepsin and a few drops of toluene. After incubation, the pH was brought to 7.0 by the addition of N/1 NaOH and the solution boiled to half its volume. It was then rendered isotonic and adjusted to pH 7.4. The ratio of total to amino nitrogen in the completed preparation was 2.8. It contained 0.32 per cent nitrogen; of this nitrogen, 36.2 per cent was present as proteose, 56 per cent as peptone, and 7.8 per cent as lower split products.[2] There was no meta-protein present. About 7.8 per cent of the nitrogen was precipitated by 2 per cent trichloracetic acid. This fraction has been classed as proteose[3] rather than as protein, although

[1] Carrel, A., and Baker, L. E., *J. Exp. Med.*, 1928, xlvii, 353.

[2] Duplicate preparations vary to a slight extent. All of them, except one which contained considerable fatty material and a suspension of heat coagulated material too fine to centrifuge out, have supported a large growth of sarcomatous fibroblasts.

[3] It is believed that the substances precipitated by 2 per cent of trichloracetic acid cannot be classed as protein, but rather as proteose, for the authors obtain

Wasteneys and Boorsook, whose method of analysis was employed, are of the opinion that it should be classed as protein.[4] Before being used in the culture medium, this digest was diluted with 7 parts Tyrode solution, making the concentration of nitrogen 0.04 per cent.

The pituitary digest[5] was prepared by incubating 12 gm. of the finely ground anterior lobe of pituitary glands of calves or steers in 120 cc. of 0.5 per cent pepsin in N/20 HCl for 16 hours. After digestion, the pH was brought to 7.0 and the solution boiled to half its volume, centrifuged, rendered isotonic, and finally adjusted to pH 7.4. The completed preparation contained 0.37 per cent total nitrogen, 36.3 per cent of which was present as proteose. The ratio of total to amino nitrogen was 3.4. The mixture was diluted with Tyrode solution to varying concentrations (Table I) before being used in the culture medium.

Preparation of Cultures.

The digests were tested on a pure strain of malignant fibroblasts of the rat, isolated from Crocker Foundation Sarcoma 10. The colonies were cut into two equal parts, one of which was cultivated in embryo juice as a control, and the other in the experimental fluid. Flasks 3 cm. in diameter were used. The plasma clot was formed by mixing 1 cc. of plasma previously diluted with 2 parts of Tyrode solution and a few drops of embryo juice. As soon as coagulation took place, the clot was washed for half an hour with 3 cc. of Tyrode solution and then 0.5 cc. of the experimental and control fluids was added. Every 48 hours, the cultures were washed with 3 cc. of Tyrode solution for half an hour, 0.5 cc. of nutritive fluids was added, and drawings were made, under a projectoscope, of the area of growth.

Action of the Pituitary Digest on Sarcomatous Fibroblasts.

The rates of growth of sarcomatous fibroblasts in the peptic digests of the anterior lobe of the pituitary and of their controls in embryo juice were approximately equal. Text-fig. 1 gives typical growth curves for the first passage of these tissues in a digest of calf pituitary

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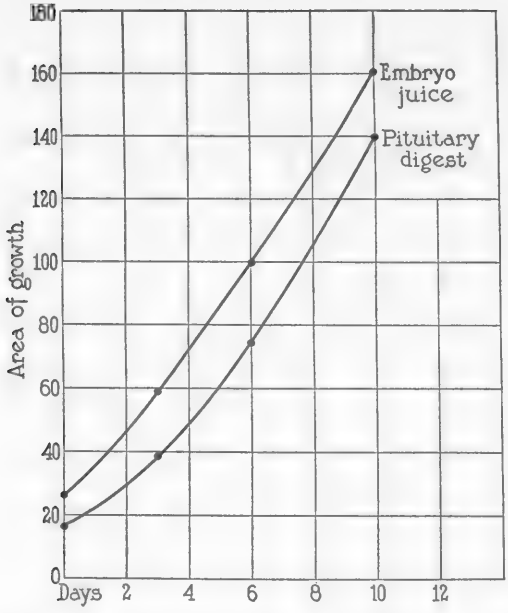
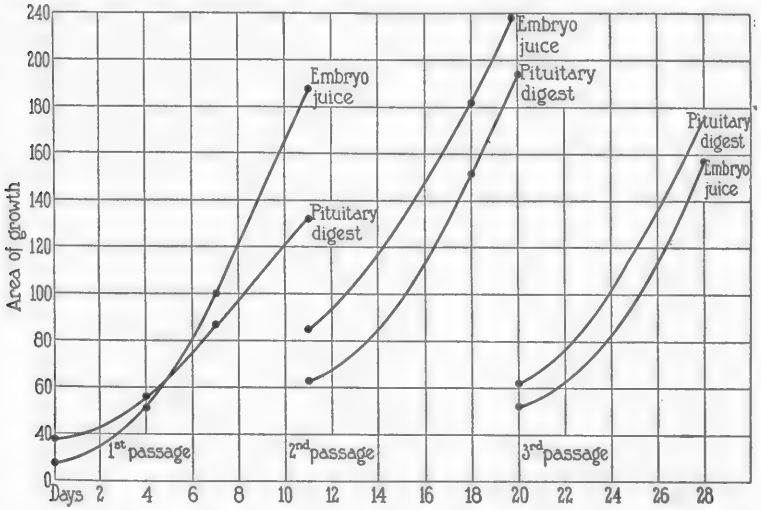
a bulky precipitate containing 10 per cent of the total nitrogen present in digests of crystalline egg albumin which have been boiled previously to remove any remaining albumin. It was also shown that this precipitate was not meta-protein, as the amount of meta-protein in the solutions was so small as to be almost negligible. Obviously, therefore, trichloracetic acid precipitates proteolytic products other than protein and meta-protein. This is in accord with the work of Hiller, A., and Van Slyke, D. D., *J. Biol. Chem.*, 1922, liii, 253.

[4] Wasteneys, H., and Boorsook, H., *J. Biol. Chem.*, 1924, lxii, 1.

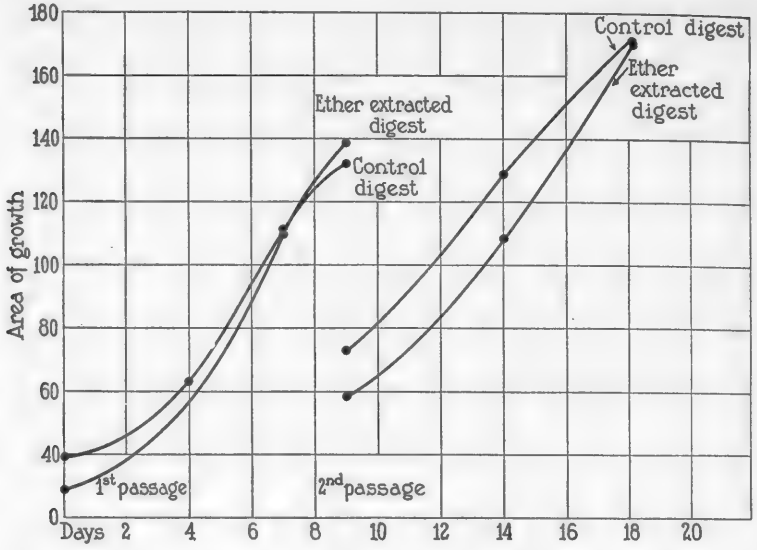
[5] Duplicate preparations of these digests have varied slightly in composition. All have, however, caused a large growth of sarcomatous fibroblasts.



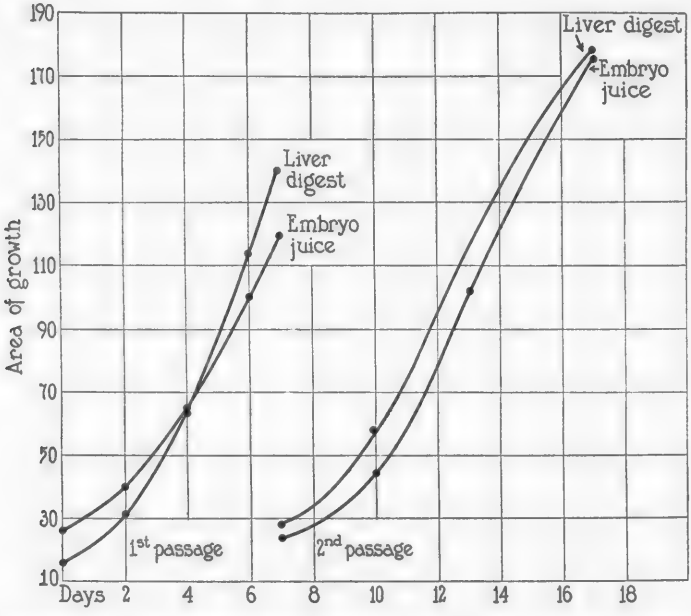
TEXT-FIG. 1. Experiment 280 H. Rate of growth of sarcomatous fibroblasts of the rat in a digest of the anterior lobe of pituitary gland, and in embryo juice.



TEXT-FIG. 2. Experiment 6211 C. Rate of growth of sarcomatous fibroblasts of the rat during three successive passages in an ether extracted digest of anterior lobe of pituitary gland. Control tissues in embryo juice.



TEXT-FIG. 3. Experiment 6130 C. Rate of growth of sarcomatous fibroblasts of the rat in a digest of the anterior lobe of the pituitary and in the same digest extracted ten times with ether.



TEXT-FIG. 4. Experiments 9546 D and 9590 D. Rate of growth of sarcomatous fibroblasts of the rat during two passages in calf liver digest.

and in embryo juice. The results of sixteen experiments at different concentrations of nitrogen are summarized in Table I. Similar results were obtained with many different preparations. No differences were observed between the action of the digests of calf pituitary and of those of steer. Not only was the rate of growth in the pituitary digest as great as in embryo juice, but the cells remained in excellent

TABLE I.

Comparison of the Rates of Growth of Sarcomatous Fibroblasts of the Rat in Embryo Juice and in a Digest of Anterior Lobe of Pituitary.

Experiment No.	Preparation No.	Nitrogen concentration	Time of growth	Area[6] of growth in embryo juice. Control	Area[6] of growth in pituitary digest. Experiment	Ratio of area of growth in experiment to area in control	Remarks
		per cent	*days*				
280 H	x-496	0.25	10	135	124	0.91	Steer pituitary
232 H	x-496	0.25	8	63	65	1.03	" "
272 H	x-496	0.25	8	81	94	1.16	" "
4979 C	x-518	0.016	9	85	64	0.75	Calf "
4979 C	x-518	0.016	9	67	66	0.99	" "
292 H	x-518	0.032	7	82	72	0.88	" "
292 H	x-518	0.016	7	42	68	1.62	" "
4923 C	x-518	0.06	10	90	85	0.94	" "
298 H	x-518	0.20	11	127	114	0.90	" "
293 H	x-519	0.03	7	38	36	0.95	Steer "
298 H	x-519	0.20	11	115	114	0.99	" "
6143 C	x-519	0.08	8	90	88	0.98	" "
4923 C	x-519	0.25	10	66	64	0.97	" "
4923 C	x-519	0.06	10	29	52	1.79	" "
320 H	x-519	0.03	12	78	65	0.83	" "
5031 C	x-513	0.03	9	61	62	1.02	Calf "
Average ratio.....						1.04	

condition as far as could be observed, and did not show the fatty degeneration characteristic of cells cultivated in pure protein digests. The same colonies continued to proliferate through many successive passages at a uniform rate. Text-fig. 2 shows three successive passages of a colony in a digest that had been thoroughly extracted with

[6] This is the area in square centimeters of the projected image of the colony of cells, and is 62 times the size of the colony.

ether. It was thought that possibly the lipoids of the glands were furnishing some substance required by the cells which might explain, in part at least, the difference between digests of pure proteins and those of glandular material,[7] but evidently this is not the case. The cells multiplied as rapidly in digests of the pituitary which had been extracted ten times with ether as in embryo juice (Table II), or in a part of the same digest which was not extracted (Text-fig. 3, Table III).

TABLE II.

Rates of Growth of Sarcomatous Fibroblasts of the Rat in Ether Extracted Digest of Anterior Lobe of Pituitary and in Embryo Juice.

Experiment No.	Preparation No.	Nitrogen concentration	Time of growth	Area[8] of growth in embryo juice. Control	Area[8] of growth in pituitary digest. Experiment	Ratio of area of growth in experiment to area in control	Remarks
		per cent	*days*				
6132 C	x-485	0.03	8	118	92	0.78	1st passage
6158 C	x-485	0.03	10	104	107	1.03	" "
6115 C	x-484	0.03	11	86	86	1.00	" "
6115 C	x-484	0.03	9	No control	93		2nd "
6115 C	x-484	0.03	8	" "	96		3rd "
6115 C	x-484	0.03	6	" "	57		4th "
6211 C	x-484	0.06	11	160	100	0.63	1st "
6211 C	x-484	0.06	10	145	130	0.90	2nd "
6211 C	x-484	0.06	9	127	114	0.90	3rd "
Average ratio.....						0.87	

Action of the Liver Digests on Sarcomatous Fibroblasts.

The digests of liver gave even better results. In many cases, the rate of multiplication of the sarcomatous fibroblasts in the liver digest exceeded that of their controls in embryo juice (Table IV). The growth remained uniform throughout many successive passages (Text-fig. 4), with no change that could be noticed in the condition of the cells. A strain of these fibroblasts was kept in this medium for over 3 months, *i.e.*, through ten passages, and was still proliferating as actively as those which had been cultivated in embryo juice.

[7] The experiments reported in the preceding paper[1] indicate that the presence of nucleic acid in digests of glandular material accounts in part for this difference.

TABLE III.

Rates of Growth of Sarcomatous Fibroblasts of the Rat in a Digest of the Anterior Lobe of Pituitary and in the Same Digest Extracted with Ether Ten Times.

Experiment No.	Preparation No.	Nitrogen concentration	Time of growth	Areas of growth in pituitary digest. Control	Areas of growth in ether extracted digest. Experiment	Ratio of area of growth in experiment to area in control	Remarks
		per cent	*days*				
6130 C	x-556	0.04	9	104	99	0.95	1st passage
6130 C	x-556	0.04	9	99	115	1.16	2nd "
6133 C	x-556	0.02	9	77	95	1.23	1st "
9424 D	x-556	0.02	6	29	28	0.96	" "
9444 D	x-556	0.04	8	56	50	0.89	" "
Average ratio.....						1.04	

TABLE IV.

Rate of Growth of Sarcomatous Fibroblasts of the Rat in Calf Liver Digest. Controls Cultivated in Embryo Juice.

Experiment No.	Preparation No.	Nitrogen concentration	Time of growth	Areas of growth in embryo juice. Control	Areas of growth in liver digest. Experiment	Ratio of area of growth in experiment to area in control	Remarks
		per cent	*days*				
9513 D	x-583	0.04	17	180	162	0.90	1st passage
9513 D	x-583	0.04	9	170	100	0.59	" "
9623 D	x-583	0.04	7	38	80	2.11	2nd "
9667 D	x-583	0.04	5	100	50	0.50	3rd "
9530 D	x-583	0.08	12	118	144	1.22	1st "
9622 D	x-583	0.08	7	57	79	1.39	2nd "
9666 D	x-583	0.08	5	96	113	1.18	3rd "
9546 D	x-583	0.08	7	92	129	1.40	1st "
9590 D	x-583	0.08	10	157	153	0.97	2nd "
9590 D	x-583	0.08	10	126	151	1.20	" "
9631 D	x-583	0.08	6	51	87	1.71	3rd "
9631 D	x-583	0.08	6	48	100	2.08	" "
9659 D	x-583	0.08	6	37	109	2.95	4th "
Average ratio						1.40	

Action of Liver and Pituitary Digests on Normal Fibroblasts.

When pure strains of normal chicken and normal rat fibroblasts were cultivated in the liver and pituitary digests, considerable proliferation of cells occurred. The rate of growth was not as great as in embryo juice and the cells finally underwent fatty degeneration. The normal tissue appears to be more sensitive to the deficiencies of the medium than the sarcoma tissue. Further experimentation is necessary before conclusions can be drawn concerning this difference in normal and sarcomatous cells.

SUMMARY.

1. A media containing all the essential constituents for the cultivation *in vitro* of sarcomatous fibroblasts of the rat has been prepared by digesting calf liver and also the anterior lobe of the pituitary body with pepsin.

2. The nutritive action of the pituitary digests is not altered by thorough extraction with ether.

3. After a pure strain of sarcomatous fibroblasts had been cultivated for 3 months in a liver digest, its proliferative activity was as great as at the beginning of the experiment. The same was true of the colonies cultivated for 1 month in a digest of pituitary gland. The increase in the volume of the colonies which takes place in the digests is about as great as that produced by chick embryo juice.

4. Normal chicken fibroblasts also proliferate in both digests, but they undergo fatty degeneration after a more or less prolonged period of cultivation.

ON THE RÔLE OF CARBOHYDRATE HAPTENS IN BACTERIAL ANAPHYLAXIS.

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Though the existence of bacterial anaphylaxis was demonstrated by Rosenau and Anderson (1) as early as 1907, subsequent investigators could not diminish the difficulties in producing a clear-cut anaphylactic shock with the majority of microorganisms. The main difficulty in the study of bacterial anaphylaxis is the primary toxicity of the bacterial protein. According to Doerr (2) the amount of bacterial antigen which produces shock in sensitized animals is negligibly smaller than the lethal dose for normal animals. More recently Zinsser and Parker (3) found that in guinea pigs sensitized passively with typhoid immune serum at least a minimal fatal dose of the typhoid extract is required to elicit in sensitized animals acute shock with death.

In the study of bacterial anaphylaxis many investigators have used suspensions of organisms, not extract, so the *in vivo* tests are complicated by the fact that these suspensions may produce symptoms even in untreated animals. This complication can be obviated by using the Schultz-Dale method. Sherwood and Stoland (4) working with typhoid bacilli found that in some cases the sensitized uterus contracted after the addition of 0.25 cc. of bacterial extract, whereas the minimum toxic dose was 1 cc. Since the uteri of the sensitized animals failed to react in many cases, they conclude, on the basis of comparative examinations, that more positive results can be obtained with the *in vivo* method and that the Dale test is not a necessary criterion of sensitization. Zinsser and Mallory (5) do not share this unfavorable opinion. They find that uterine reactions, like those obtained after sensitization with proteins, can be demonstrated in guinea pigs after both active and passive sensitization against alkaline extracts of pneumococci, but "The margin between the minimum doses which contract the normal and those which contract the sensitized uteri is incomparably smaller than that obtained in analogous experiments with . . . . egg albumen." With regard to the difficulty of demonstrating bacterial anaphylaxis Zinsser and Mallory state: "it would seem to us that active sensitization is so difficult because the bacteria contain relatively little coagulable protein, and that in passive sensitization there is an apparently slower union of antibodies with the animal tissues than in the case of protein antibodies." If this assumption is right we have little hope of improving our methods for the study of bacterial anaphylaxis, the difficulties being inherent in the nature of this phenomenon. It is conceivable however, that the primary toxic substance

of the bacteria is not necessarily identical with the specific antigenic part and that by suitable chemical separation it may be possible to isolate an atoxic specific constituent, responsible for the production of anaphylactic shock. With the discovery of specifically reacting polysaccharides in certain microorganisms a new field was opened for investigation. These substances, immunologically haptens, give powerful precipitation and complement fixation reactions with immune serum, but they do not lead to the formation of antibodies, when injected into animals. According to Doerr the anaphylactic antibodies against bacterial proteins are probably identical with the precipitins, since the power of an immune serum to confer passive sensitization is in proportion to its precipitin content. If this is true we should be able to produce anaphylactic shock with a carbohydrate hapten, since it has a remarkably high activity in precipitin reactions.

As far as we are aware no experiments have been reported which show a difference between the sensitizing and shock-producing part of the antigen in typical anaphylaxis. However in the classical investigations of Zinsser and his associates it appears that such a difference exists in the related field of bacterial allergy. Zinsser and Parker (6) produced typical skin reactions with residue antigen in guinea pigs infected with tuberculosis. In later work Zinsser and Mueller (7), then Zinsser and Tamiya (8) succeeded in isolating from *Bacillus tuberculosis* and the pneumobacillus an active material which produced a positive skin test and yet appeared to be different from the soluble specific substance. Both of these substances lack true antigenic activity, the substance producing skin reactions being a protein-free nitrogenous material, while the soluble specific substance is a carbohydrate. This work was not extended to the study of typical anaphylaxis, probably because it was a generally accepted opinion that true protein anaphylaxis plays an unimportant rôle in the occurrence of spontaneous infections.

The object of the present work was to determine whether animals could be sensitized to carbohydrate haptens, either actively by the injection of bacteria, or passively by immune serum. Sensitization has been determined by the injection of the hapten intravenously and by the reaction of the uterus according to the Schultz-Dale technic.

Bacillus lactis aerogenes was chosen for the major part of our study. The isolation of the specific substance from this bacterium and its chemical nature has been described by one of us previously (9). It consists chiefly of carbohydrates containing, after hydrolysis, 66 per cent reducing substance, counted as glucose. It is protein-free, but in spite of several attempts at further purification, its nitrogen content could not be reduced below 0.9 per cent. It gave specific precipitation reaction, when diluted in 1:500,000 and complement fixation reaction when diluted as high as 1:64,000,000.

EXPERIMENTAL.

In Vivo Tests in Actively Sensitized Animals.

Attempts were made at the beginning of our work to produce active sensitization against the *aerogenes* specific substance.

Holobut's method (10) was adopted in sensitizing the animals, using massive intraperitoneal inoculations of living or killed organisms on 10 consecutive days and testing the sensitivity 3 weeks after the last injection. The difficulty we encountered was the high toxicity of the bacillus for guinea pigs. In the first lot of 12 guinea pigs, 6 were inoculated with living bacteria and 6 with bacteria killed at 60°C. Each animal died after the second or the third injection, when the single dose was 1/20th of the growth from a 24 hour agar culture. In inoculating the second lot of guinea pigs, smaller doses were used, beginning with 1/200th and slowly increasing to 1/60th part of a 24 hour agar culture. 6 guinea pigs were inoculated with living and 6 with killed bacteria and daily injections were given on 9 consecutive days. At the end of this treatment 3 animals survived from each group. These were tested for sensitivity 3 weeks after the last injection, in part by intravenous inoculation of 1 mg. aerogenes specific substance, in part by testing the isolated uterus. No reaction was observed in any of these tests.

Passive Sensitization toward the Aerogenes Specific Substance.

Our experiments in regard to active sensitization were not extensive enough for the formulation of any conclusion. In view of the difficulty of obtaining sensitization in this way we limited ourselves in further work to the study of passive anaphylaxis. One part of these experiments was reported by one of us in a preliminary paper (11).

The immune serum we have used for the passive sensitization was prepared in rabbits by six to eight intravenous injections of the killed suspension of *Bacillus lactis aerogenes*. The interval between the injections was either 3 or 4 days. The rabbits were bled 8 days after the last injection.

In performing the sensitization, guinea pigs weighing 250 to 380 gm. were inoculated intraperitoneally with from 1 to 4 cc. immune serum. After 16 to 24 hours, 1 cc. of the various dilutions of the specific substance in saline was injected in the saphenous vein. Each of the 18 sensitized guinea pigs tested intravenously with 1 to 0.033 mg. of the specific substance died, showing typical anaphylactic symptoms. Death usually occurred within 2 to 3 minutes after the injection and never later than 5 minutes. The lungs were markedly distended and pale. 1 guinea pig receiving 0.02 mg. specific substance showed symptoms but survived.

Control tests were made on 11 untreated guinea pigs weighing 250 to 300 gm. None of these animals showed any immediate symptoms. When the dose was as

high as 1 to 2 mg. some of the animals died on the 2nd or 3rd day, without showing any symptoms other than drowsiness and emaciation. At the autopsy no changes were observed. Smaller amounts than 1 mg. never caused any symptoms in untreated animals. The slight toxicity of the aerogenes specific substance in normal animals can be explained probably by the presence of a small amount of nitrogenous material. The margin between the primary toxic dose (2 mg.) and the amount which produced a fatal shock (0.033 mg.) was much wider than that found previously with bacterial extracts.

It should be noted that there was not a single experiment in which we failed to produce a fatal shock in sensitized animals, provided the amount of the extract inoculated intravenously was not less than 0.033 mg.

In tests to confirm the results obtained *in vivo*, the smooth muscle reaction was used in further work. 20 guinea pigs weighing 210 to 260 gm. were sensitized by the intraperitoneal injection of 1 cc. aerogenes immune serum and the responses of their uteri tested, using the Schultz-Dale method. The uteri of all these animals reacted to the specific substance, when tested 2 hours to 12 days after the injection of serum. According to earlier authors the optimal incubation time for the development of passive anaphylaxis is about 1 day. Zinsser and Parker (3) in their experiments with typhoid bacilli never succeeded in finding the animals sensitized in less than 3 to 5 days, the highest degree of sensitization being developed in 1 week. Using the aerogenes specific substance we did not observe appreciable difference in the sensitivity even when the incubation time varied between 2 hours and 12 days. In establishing accurately the smallest amount of specific substance which causes a contraction in the sensitized uterus, one difficulty was observed, apart from the individual variations of the uteri. The two horns of a uterus were suspended approximately at the same time in the two baths. To the first horn 0.5 cc. of a 1:80,000 dilution of the specific substance was added, without causing any contraction, but 1:50,000 dilution caused a distinct reaction of the other horn. After changing the bath 1:80,000 dilution added to the second horn caused a reaction with a curve similar to that following the previous larger dose. That is a slighter preliminary reaction increased the sensitivity of the uterus. This observation has been previously made by Weil (12) in his studies in protein anaphylaxis.

The smallest amount of the specific substance which caused a distinct, but not maximal contraction, was 0.00625 mg. added to a bath of 125 cc. The final concentration of the substance therefore was 1:20,000,000.

Desensitization of the uterus was demonstrated after the contraction due to one addition of 5 mg. of the substance to the bath. If, however, 1 mg. of the specific substance was used, three subsequent doses were required, when the bath was changed after each contraction, to obtain a complete desensitization.

As controls the uteri from 6 normal guinea pigs were tested and not one gave a reaction following the addition of as much as 10 mg. of the specific substance to the bath. When tested for sensitivity to specific substances obtained from other types of aerogenes, there was no response indicating overlapping specificity so that the results correspond to those obtained with the precipitin reaction.

These experiments seem to justify the conclusion that passive anaphylaxis toward a non-protein bacterial extract can be demonstrated with the same degree of sensitiveness and specificity as observed in protein anaphylaxis.

It might be mentioned briefly that in a few cases the skin test was tried in passively sensitized animals, though these experiments do not warrant any definite conclusion.

11 guinea pigs were sensitized by the intraperitoneal injection of 1 to 5 cc. immune serum. After 24 hours 0.1 cc. of 1:500 or 1:1000 dilution of the specific substance was injected into the abdominal skin. In most of these animals a moderate swelling developed in 3 to 5 hours, the largest being 2 cm. in diameter, with an inflammatory red area in the middle. The reaction disappeared in 12 to 18 hours. In 7 unsensitized animals no changes were observed in the injected area.

The type of skin reaction we observed in the sensitized animals can be classified neither as an immediate, evanescent urticarial reaction, characteristic of protein anaphylaxis, nor as a late allergic reaction, since it disappears in 24 hours. We feel that additional work is needed to establish the nature of this reaction.

Passive Sensitization to the Carbohydrate Hapten of the Pneumobacillus.

In this further work, our object was to confirm the results obtained with the aerogenes specific substance, using the carbohydrate haptens

of other microorganisms which can be purified more highly. Accordingly the specific substance was isolated from a strain of pneumobacillus, originally obtained from a normal throat.

This hapten was isolated first by Heidelberger, Goebel, and Avery (13) and after a long and careful process of purification, it was found by them to be free from nitrogen and to consist of a polysaccharide built up from glucose units. For technical reasons we could not follow the method used by these authors, but adopted Toeniessen's method (14). The raw extract thus obtained, which corresponds probably to the capsular material, was subjected to subsequent purification. At the beginning of our work the capsule of the freshly isolated strain was exceptionally abundant; 3 or 4 months later, after repeated transfer on meat infusion agar medium, it became much less. At this stage the yield of the extract was also smaller, but did not differ in reactivity from the first extract. It gave precipitation with a pneumobacillus rabbit immune serum, when diluted in 1:300,000. From the bacterial growth of 100 Kolle flasks 1.2 gm. of extract was obtained, in the form of a white powder easily soluble in water. A weakly alkaline solution of this substance in a dilution of 1:200 was precipitated 6 times in succession with 3 volumes of absolute alcohol. The resulting powder gave a specific precipitin reaction, when diluted 1:500,000 and layered over immune serum. Additional purification was carried out using the uranyl nitrate precipitation method. In this we followed very closely the technic given by Heidelberger, Goebel, and Avery (13). The resulting powder was dissolved in water and dyalized for 2 days in a parchment bag, then filtered through Seitz filter, and precipitated by alcohol. In the final stage it was precipitated by the corresponding immune serum when diluted in 1:2,000,000. It gave no precipitation with any of the pneumococcus type sera. In the presence of 1:20 dilution of the immune serum it gave complement fixation when diluted 1:32,000,000.

After hydrolysis it contained 71.5 per cent reducing substance counted as glucose. When the hydrolysis was made in normal sulfuric acid solution, the optimal time was found to be 9 hours. The nitrogen content of this specific substance was 0.26 per cent, considerably lower than that of the aerogenes extract.

The method for demonstrating anaphylaxis to the pneumobacillus carbohydrate was the same as that described above with the aerogenes specific substance.

Guinea pigs were sensitized by the intraperitoneal injection of 1 cc. pneumobacillus immune serum and 24 hours later injected intravenously with various dilutions of the specific substance. The smallest amount of the carbohydrate extract which produced invariably a typical anaphylactic shock with death was found to be 0.01 mg. 5 untreated guinea pigs were used as controls and failed to show any symptoms after the injection of amounts as large as 2 mg.

These results seem to us, to be especially convincing since the carbohydrate extract did not possess any primary toxicity and yet it produced anaphylactic shock even when given in smaller amounts than had been used previously with proteins. According to Wells (15) the smallest amount of protein capable of causing anaphylactic shock is 0.1 to 0.05 mg.

The results *in vivo* were confirmed by the Schultz-Dale reaction. A 1:12,500,000 dilution of the carbohydrate in the bath was enough to produce contractions in sensitized uteri. It is interesting that a single contraction due to the addition of 1 mg. carbohydrate to the bath was enough to desensitize the muscle completely.

Experiments with Yeast Gum.

Studies in yeast anaphylaxis have been made by Rosenau and Anderson (1) and by Axamit (16). Yeast cells or crude extracts were used in testing the sensitivity of the treated animals. The slight difference between the primary toxic and the shock-producing doses made the interpretation of these results just as difficult as in the case of bacterial anaphylaxis.

Our object was to test passively sensitized guinea pigs with yeast gum, the hapten nature of which has been shown by Mueller and by one of us (17).

The strain of yeast used in this work was obtained from a case of stomatitis. The yeast cells grown on Sabouraud culture medium were collected and freed from the constituents of the culture medium by alcoholic precipitation. The extraction was made by the hot alkaline method. From this extract the gum was obtained by the usual procedure, precipitating with Fehling reagent. The last trace of the copper was taken out by repeated alcoholic precipitation and dyalisis.

In the final stage of purity 83 per cent of the gum consisted of reducing substances counted as glucose. Nitrogen was present in trace, approximately 0.2 per cent.

Passive anaphylaxis toward the yeast gum was studied both *in vivo* and with the uterine reaction. Since the results of these experiments entirely correspond to those obtained with the specific substances of *Bacillus lactis aerogenes* and of the pneumobacillus, their description can be omitted to avoid repetition. The only difference observed was that repeated additions of larger amounts of yeast gum were

necessary to produce desensitization of the uterus, whereas this was easily accomplished with pneumobacillus extract. The injection of the yeast gum into six untreated animals proved to be without effect.

TABLE I.

Summary of the Reactions Obtained with the Specific Substances of B. lactis aerogenes, Pneumobacillus, and Yeast.

		Specific substances from		
		B. lactis aerogenes	Pneumobacillus	Yeast
Nitrogen		0.9 per cent	0.26 per cent	0.2 per cent
Reducing substances after hydrolysis, counted as glucose		66 per cent	71.5 per cent	83 per cent
Precipitin titer		1:500,000	1:2,000,000	1:1,000,000
Titer in complement* fixation		1:64,000,000	1:32,000,000	1:32,000,000
In vivo tests	Toxic dose for normal guinea pigs	1 mg.	2 mg. non-toxic	2 mg. non-toxic
	Minimal shock-producing dose	0.033 mg.	0.01 mg.	0.02 mg.
In vitro tests with Dale technic	Largest amount failing to cause contraction of normal uterus	10 mg.	10 mg.	10 mg.
	Smallest amount causing contraction of the uterus from sensitized guinea pigs	0.00625 mg.	0.01 mg.	0.025 mg.
	Amount causing desensitization	5 mg.	1 mg.	Not complete after 10 mg.

* Graded amounts of the antigens were tested in the presence of 0.5 cc. 1:20 dilution of immune serum and 2 units of complement. Incubation period was 16 hours at 6° to 8°C. The amount in the last tube which did not show a trace of hemolysis was regarded as the titer.

DISCUSSION.

The experiments described above show that small amounts of protein-free hapten obtained from *B. lactis aerogenes* will produce anaphylactic shock, both *in vivo* and *in vitro*, in guinea pigs which have been passively sensitized with immune serum from rabbits injected

with dead cultures of the same organism. In the same way, haptens from the pneumobacillus and from a yeast caused typical shock in animals sensitized by the appropriate immune sera. When haptens are used to bring out the reaction, the wide margin between the toxic and the minimal shock-producing doses corresponds to that found in protein anaphylaxis. Desensitization is produced in the isolated uterus by the addition of the hapten to the bath. The muscle is of course washed and rested before the desensitization test is made.

Repeated injections of living and killed cultures have not produced a sensitization to the hapten in the few animals that survived the treatment.

Just as haptens fail to cause the production of precipitins and complement-fixing bodies, so do they fail to sensitize guinea pigs when injected in their pure state. That they may play a part in sensitization when combined with protein seems possible.

The haptens used in these experiments gave none of the protein reactions, and while they were not absolutely nitrogen-free, this element was almost negligible in the pneumobacillus and yeast preparations. Since shock was produced by smaller amounts of hapten than will cause a reaction in animals sensitized to purified proteins, it does not seem possible that the small amount of nitrogen present played any part in the reaction. A positive statement is unjustified because of the trace of nitrogen present, but we believe that we have shown in these experiments that anaphylactic shock can be produced by carbohydrates.

Since the serum used for the sensitizations had high contents of precipitin and complement-fixing bodies which react with minute amounts of the appropriate hapten, we feel that the results obtained support the view that precipitins and complement-fixing bodies are closely related to the bodies which have to do with sensitization, if not identical with them.

CONCLUSIONS.

1. In passively sensitized animals bacterial anaphylaxis has been produced, *in vivo* and *in vitro*, with haptens from *B. lactis aerogenes*, the pneumobacillus, and a yeast.

2. The smallest amount of hapten causing fatal anaphylaxis is less

than the minimal amount of protein which will cause death in properly sensitized animals.

3. The haptens used were largely carbohydrates, and gave none of the protein reactions, but since they did contain a small amount of nitrogen we cannot yet assert positively that carbohydrate alone will produce shock.

4. Since haptens will not sensitize animals we must conclude that the anaphylactogenic and shock-producing parts of the antigen are not identical.

5. These experiments provide further evidence of the close relationship of precipitins to anaphylaxis.

BIBLIOGRAPHY.

1. Rosenau, M. J., and Anderson, J. F., *Bull. Hyg. Lab., U. S. P. H., No. 36*, 1907.
2. Doerr, R., in Kolle, W., and von Wassermann, A., Handbuch der pathogenen Mikroorganism, Jena, 2nd edition, 1913, ii, 947.
3. Zinsser, H., and Parker, J. T., *J. Exp. Med.*, 1917, xxvi, 411.
4. Sherwood, N. P., and Stoland, O. O., *J. Immunol.*, 1925, x, 643.
5. Zinsser, H., and Mallory, T., *J. Immunol.*, 1924, ix, 75.
6. Zinsser, H., and Parker, J. T., *J. Exp. Med.*, 1923, xxxvii, 275.
7. Zinsser, H., and Mueller, J. H., *J. Exp. Med.*, 1925, xli, 159.
8. Zinsser, H., and Tamiya, T., *J. Exp. Med.*, 1925, xlii, 311.
9. Tomcsik, J., *Proc. Soc. Exp. Biol. and Med.*, 1927, xxiv, 810.
10. Holobut, P., *Z. Immunitätsforsch., Orig.*, 1909, iii, 639.
11. Tomcsik, J., *Proc. Soc. Exp. Biol. and Med.*, 1927, xxiv, 812.
12. Weil, R., *J. Med. Research*, 1914, xxx, 87.
13. Heidelberger, M., Goebel, W. F., and Avery, O. T., *J. Exp. Med.*, 1925, xliii, 701.
14. Toeniessen, E., *Centr. Bakt., 1. Abt., Orig.*, 1921, lxxxv, 225.
15. Wells, H. G., The chemical aspects of immunity, American Chemical Society Monograph Series, New York, 1925.
16. Axamit, O., *Arch. Hyg.*, 1907, lxii, 15.
17. Mueller, J. T., and Tomcsik, J., *J. Exp. Med.*, 1924, xl, 343.

STUDIES ON THE INACTIVATION OF VACCINE VIRUS AND THE ACTION OF CERTAIN SUBSTANCES UPON THE INFECTING POWER OF THE INACTIVATED VIRUS.*

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The present status of the discussion opened by the experiments of Gye and Barnard (1, 2) supporting the virus theory of cancer, can be summarized as follows. While certain investigators have been able to repeat Gye's results and are in agreement with his interpretations (3, 4), others have failed (5–8). Murphy (9) and more recently Flu (10) and Cori (11) duplicated Gye's work but by means of more careful control experiments have added a new fact of basic importance, namely the non-specific reactivation of the chloroformed sarcoma filtrate (specific factor) by substances other than the cultures of malignant tissues. It is obvious that Gye's theory does not hold in the light of these later facts, although Gye himself claims to get only negative results in 150 control experiments of a similar nature.

In the opinion of Murphy also shared by Flu, the interpretation of these results is not that a virus has been rendered infective by a specific factor but that an unknown substance of tissue origin enables the agent, modified or attenuated by chloroform, to act. Flu suggests that these substances are similar to bacterial aggressins. Simon and Beck (6) explained Gye's results on the basis of an aggregation of subinfective doses of the agent itself or of a non-specific reactivation, while Harde (12) suggests that the activator is effective only through its acidity.

In order to throw further light on this subject we have carried out

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an investigation similar to that of Gye but have substituted a typical virus in the place of the etiological agent of the chicken sarcoma. Our plan has been to determine whether the vaccine virus attenuated to such an extent that it would produce no lesion could be rendered infective by the addition of certain substances.

Methods and Materials.

Vaccine Virus.—In all of the experiments the same strain of vaccine virus has been used.[1] Half a cc. of the vaccine emulsion mixed with an equal amount of Ringer's solution was injected into both testicles of a rabbit. Five days later when the resulting orchitis was at its height the animal was killed and the testicles removed

TABLE I.

Tube	Amount of chloroform	Resulting lesion	Tube	Amount of ether	Resulting lesion
	cc.			*cc.*	
1	0.2	−	4	0.2	++
2	0.15	−	5	0.15	++
3	0.1	+	6	0.1	++

TABLE II.

Tube	Amount of chloroform	Resulting lesion	Tube	Amount of ether	Resulting lesion
	cc.			*cc.*	
1	0.15	−	5	0.2	++
2	0.1	±	6	0.15	++
3	0.05	+	7	0.05	+++
4	0.01	+			

aseptically. They were ground thoroughly with sand together with 25 cc. of glycerol and Ringer's solution. The resulting emulsion was distributed in tubes, covered with a layer of sterile vaseline and kept in the ice box.

In order to test the activity of the virus the emulsion was diluted with Ringer's solution and 0.2 cc. injected intradermally in rabbits. With a 1 to 500 dilution of the emulsion (about 1 to 5000 of the testicular material) a slight but definite

[1] We wish to acknowledge our indebtedness to Dr. T. M. Rivers for supplying the original strain of vaccine virus and for his suggestions and criticisms during the course of this investigation.

vaccine eruption appeared in 4 or 5 days. The injection of 0.2 cc. of a 1 to 10 dilution gave rise in the same time to a greatly congested circular eruption 2.5 to 3 cm. in diameter. This latter dose was the one generally used in the experiments.

Inactivation of Virus.

The same general methods have been employed as were used in Gye's experiments. Not only chloroform but other organic solvents were tested as to their effect on the vaccine virus.

Method.—To 10 cc. of the vaccine emulsion diluted 1 to 10 and placed in a 50 cc. centrifuge tube, various amounts of one or another of the solvents were added.

TABLE III.

Tube	Amount of acetone	Lesion	Tube	Amount of acetone	Lesion
	cc.			*cc.*	
1	0.5	++	5	0.15	++
2	0.5	++	6	0.1	++
3	0.3	++	7	Control	++
4	0.2	++			

TABLE IV.

Tube	Amount of chloroform	Lesion	Tube	Amounts	Resultant lesion
	cc.			*cc.*	
1	0.2	−	4 alcohol	0.2	+++
2	0.15	−	5 toluene	0.2	+++
3	0.1	±	6 control		+++

These included chloroform, ether, 95 per cent alcohol and toluene. The materials were thoroughly mixed by means of a pipette and were incubated at 37°C. for from 75 to 90 minutes. After this the solvents were evaporated off in a vacuum and the activity of the treated virus was tested by injection into the skin of freshly shaved rabbits.

Experiment 1.—The effect of chloroform and ether in 0.2, 0.15 and 0.1 cc. amounts on 10 cc. of vaccine emulsion was tested. The mixtures were incubated at 37°C. for 65 minutes, after which the solvents were evaporated off and 0.2 cc. of the remaining virus from each tube was injected intradermally into rabbits. The results are shown in Table I.

Experiment 2.—The same procedure as in Experiment 1 was carried out except

that the mixtures were incubated for 90 minutes. The results are shown in Table II.

Experiment 3.—The action of acetone was tested by the same method as described above, the mixtures being incubated for 90 minutes. The results are shown in Table III.

Experiment 4.—Additional tests were made with chloroform, 95 per cent alcohol and toluene, the mixtures being incubated for 75 minutes. The results are given in Table IV.

The above results show that the vaccine virus is very susceptible to the action of chloroform and the action seems to grade off as the dilution is reduced. Generally 0.2 cc. will inactivate 10 cc. of the emulsion so that no lesion results from its injection. When 0.15 cc. of chloroform was used, the virus was inactive in doses of 0.2 cc. Subsequent tests have shown that when as much as 1 cc. of this virus is injected a definite but small eruption appeared in from 10 to 12 days. The injection of still larger amounts, from 5 to 10 cc. gave rise to lesions almost equivalent in severity and duration to that produced by 0.2 cc. of the untreated virus. It should be noted that there is naturally some variation in the results as the amount of the virus present in the testicular emulsion must be subject to considerable variation, as well as the natural susceptibility of the animals.

Ether, 95 per cent alcohol, acetone and toluene in amounts comparable to the amounts of chloroform found to be effective seem to have little or no action on the virus.

Reactivation of Chloroformed Virus.

With the fact demonstrated that vaccine virus is sensitive to chloroform, the next step was to determine whether the inactive or very slightly active virus could be so influenced by the addition of other agents that it would become infective in small doses. For the secondary agents, we used those found effective in activation of the chloroformed filtrates of the chicken tumor.

Preparation of Auxiliary Agents.—Chicken embryos 7 to 10 days old were obtained aseptically after careful disinfection of the shell of the egg, and were placed in tubes containing 5 cc. of Hartley's broth with glucose, potassium chloride and rabbit serum. These were incubated at 37°C. under anaerobic conditions from 4 to 25 days. After removing the tubes they were shaken in order to mix the

tissue with the fluid and were then allowed to stand or were centrifuged in order to obtain a more or less clear fluid. Sterility was tested either by smears or subcultures.

The same procedure was carried out using fragments of chicken sarcoma instead of embryos.

The third substance used in the experiments was a light suspension of kieselguhr in distilled water.

Effect of Embryo "Cultures" and Small Amounts of Chloroformed Virus.—In the first series of experiments, carried out on ten rabbits, the action was studied of a mixture of supernatant fluid of embryo "cultures" with small amounts of chloroformed virus (0.2 cc.), namely, the same amount which untreated gives rise to a typical vaccine eruption in 4 to 5 days. The results were negative as regards a vaccinal lesion. Nevertheless, the experiments brought out some interesting facts. At the end of the first 24 hours, occasionally, but generally in 2 days, there appeared a circular zone of erythema and edema, similar in dimensions to the true vaccine eruption, but more diffuse and less indurated. This increased during the 3rd day, then ceased to spread and finally disappeared more quickly than the typical vaccine eruption. Without proper control experiments, namely injection of the embryonic fluid alone, this eruption might have been the source of considerable misunderstanding. The irritating power of the embryo culture fluid proved to be weak if the cultures were young (3 to 4 days), strong if the cultures were older. The irritation is greater if the supernatant fluid to be injected contains tiny bits of embryonic tissue in suspension.

Effect of Chicken Sarcoma "Cultures" or Kieselguhr Together with Small Amounts of Chloroformed Vaccine Virus.—Some experiments were carried out with small amounts of vaccine virus, such as those mentioned above, the reactivating substance being the supernatant fluid of chicken sarcoma "cultures." These experiments were few in number, but, in general, the results justify the conclusion that these "cultures" by themselves are capable of producing in rabbits similar reactions to those produced by the supernatant fluid of embryo "cultures."

Kieselguhr produces, when injected intracutaneously, a very wide reaction, resembling the reaction caused by the vaccine virus even

more than do those produced by embryo "cultures." This point will be discussed in greater detail further on in the paper.

Effect of Embryo "Cultures" Kieselguhr Together with Larger Amounts of Chloroformed Vaccine Virus.—After the reactions induced by the embryo "cultures" had been studied and differentiated from true vaccine eruptions, the experiments were repeated with larger doses of the chloroformed vaccine and of the "culture" fluid. We further tested the activity of larger doses of the chloroformed vaccine alone keeping in mind Flu's statement that he could obtain reactivation of the chloroformed sarcoma agent only when he got tumors from the injection of large amounts of the chloroformed filtrate alone.

Experiment 1.—To 10 cc. of the vaccine virus emulsion diluted 1 to 10 with Ringer's was added 0.15 cc. of chloroform. After mixing well the tube was incubated at 37°C. for 78 minutes. The chloroform was removed in a vacuum. For the secondary substances the slightly turbid supernatant fluids from 18 day old "cultures" of embryonic tissue in Hartley's broth, and a light suspension of kieselguhr were used. The nature of the intradermal injections and the intensity of the vaccinal eruptions induced were as follows.

Rabbit 1.

		Maximum intensity of eruption
1. Chloroformed vaccine virus 0.5 cc. plus supernatant fluid embryo "culture" 0.5 cc.	Lesion +	10 days
2. Chloroformed vaccine virus 1 cc. plus supernatant fluid embryo "culture" 1 cc.	Lesion ++	10 days
3. Chloroformed vaccine virus 0.5 cc. plus light suspension kieselguhr 0.5 cc.	Lesion ++	10 days
4. Supernatant fluid embryo "culture" 0.5 cc.	No lesion	
5. Supernatant fluid embryo "culture" 1 cc.	No lesion	
6. Kieselguhr suspension 0.3 cc.	No lesion	

Rabbit 2.

1. Chloroformed vaccine virus alone 0.5 cc.	No lesion	
2. Chloroformed vaccine virus alone 0.5 cc.	No lesion	
3. Untreated vaccine virus 0.2 cc.	Lesion +++	5 days

Rabbit 3.

1. Chloroformed vaccine virus alone 5 cc.	Lesion ++	6 days

Part of the lesion produced by the mixture of kieselguhr and chloroformed vaccine virus was removed and injected into a susceptible animal (rabbit) and a positive vaccinal eruption was obtained. All rabbits of this experiment were tested

2 months later by a second inoculation of fresh vaccine virus and found to be immune, an indication that the lesions produced were true vaccinal eruptions.

Experiment 2.—This was similar to the foregoing experiment except that 24 day old "cultures" of embryonic tissue were used.

Rabbit 1.

		Maximum intensity of eruption
1. Chloroformed vaccine virus 0.5 cc. plus supernatant fluid embryonic "culture" 0.5 cc.	Lesion −	
2. Chloroformed vaccine virus 1 cc. plus fluid embryonic "culture" 0.5 cc.	Lesion ++	8 days
3. Chloroformed vaccine virus 0.5 cc. plus kieselguhr suspension 0.4 cc.	Lesion ++	8 days
4. Supernatant fluid embryonic "cultures" 0.5 cc.	No lesion	
5. Supernatant fluid embryonic "cultures" 1 cc.	No lesion	

Rabbit 2.

1. Untreated vaccine virus 0.2 cc.	Lesion ++	5 days
2. Untreated vaccine virus 0.4 cc.	Lesion +++	5 days
3. Chloroformed vaccine virus 0.5 cc.	No lesion	
4. Chloroformed vaccine virus 1 cc.	No lesion	

Rabbit 3.

5. Chloroformed vaccine virus 5 cc.	Lesion ++	5 days

The skin of the spot injected with 1 cc. of chloroformed vaccine virus alone, which did not show a definite eruption, was removed after 5 days and injected, after grinding, into the testicles of another rabbit. It gave rise to an orchitis. From this testicle a virus was obtained which gave rise to a typical pustular eruption in the skin of another animal. All rabbits of this experiment were injected 2 months later with fresh vaccine virus and found to be immune.

The above two experiments show that chloroformed vaccine virus in 0.5 and 1 cc. doses do not give rise to eruptions when injected alone into the skin of a rabbit, but the addition of the supernatant fluid of embryonic tissue "cultures" or a suspension of kieselguhr results in very definite and typical lesions. The same degree of eruption results from the injection of larger amounts of the chloroformed virus alone (5 to 10 cc.). Occasionally small amounts gave rise to eruptions so slight as to leave doubt as to their nature but for the fact that the virus may be recovered from such lesions in animal passage. The eruption which takes place as the result of the injection of a mixture

of chloroformed virus and the secondary agent, comes later than that produced by the untreated virus or the larger doses of chloroformed virus.

In the next group of experiments care was taken to test the untreated vaccine virus and the chloroformed virus on different animals in order to avoid possible immunity effects. It was thought that the reaction following the pronounced eruption from the untreated virus might interfere with a weaker delayed reaction from the treated virus.

Experiment 3.—The general procedure of this experiment was the same as the foregoing. A 39 day old "culture" of 7 day old chick embryo tissue was used as the secondary factor and the vaccine virus was exposed to the action of chloroform for 70 minutes at 37°C. The results were as follows.

Rabbit 1.

		Maximum intensity of eruption
1. Chloroformed vaccine virus 0.5 cc. plus embryo "culture" fluid 0.5 cc.	Lesion +	8 days
2. Same as above	Lesion ++	9 days
3. Chloroformed vaccine virus 0.5 cc. plus 0.5 cc. kieselguhr suspension	Lesion −	
4. Chloroformed vaccine virus alone 0.5 cc.	Lesion −	
5. Fluid from embryo "culture" 0.5 cc.	Lesion −	
6. Kieselguhr suspension 0.5 cc.	Lesion −	

Rabbit 2.

1. Chloroformed vaccine virus 5 cc.	Lesion ++	6 days
2. Chloroformed vaccine virus 0.5 cc.	Lesion −	
3. Chloroformed vaccine virus 0.5 cc.	Lesion −	

Rabbit 3.

1. Untreated vaccine virus 0.2 cc.	Lesion ++	5 days
2. Untreated vaccine virus 0.4 cc.	Lesion +++	5 days

One of the spots of the skin of Rabbit 2 where 0.5 cc. of chloroformed vaccine virus had been injected was removed and injected, after grinding with sand, into the testicle of a normal rabbit. An orchitis developed from which a virus was obtained which regularly gave a typical vaccinal eruption in susceptible animals.

All of the rabbits of this experiment were tested 2 months later by a second inoculation of fresh untreated vaccine virus and all proved to be immune.

Experiment 4.—The same general procedure was followed here as in the foregoing experiment except that 0.2 instead of 0.15 cc. of chloroform was added to the vaccine virus. The results were as follows.

Rabbit 1.

		Maximum intensity of eruption
1. Chloroformed vaccine virus 0.5 cc. plus supernatant fluid embryonic "culture" 0.5 cc.	Lesion +	8 days
2. Chloroformed vaccine virus 0.5 cc. plus supernatant fluid embryonic "culture" 0.5 cc.	Lesion −	
3. Chloroformed vaccine virus 0.5 cc. plus kieselguhr dilution 0.5 cc.	Lesion −	
4. Chloroformed vaccine virus alone 0.5 cc.	Lesion −	
5. Supernatant fluid embryonic "culture" 0.5 cc.	Lesion −	
6. Supernatant fluid embryonic "culture" 0.5 cc.	Lesion −	
7. Kieselguhr dilution alone	Lesion −	

Rabbit 2.

1. Chloroformed vaccine virus alone 5 cc.	Lesion −	
2. Chloroformed vaccine virus alone 0.5 cc.	Lesion −	
3. Chloroformed vaccine virus alone 0.5 cc.	Lesion +	8 days

Rabbit 3.

1. Untreated vaccine virus 0.2 cc.	Lesion ++
2. Untreated vaccine virus 0.4 cc.	Lesion +++

All the animals of this experiment were tested with fresh vaccine virus 2 months later and found immune.

These last two experiments indicate that the immunity possibly developing as the result of the stronger reaction to untreated vaccine did not influence the results.

The last experiment cannot be considered as entirely a satisfactory result for 0.5 cc. of chloroform vaccine alone caused a slight lesion in the control rabbit but gave a negative reaction in the test animal. The indications are that the greater the amount of chloroform used the less is the possibility of reactivating the virus. In order to get some idea of the limits the following experiments have been carried out. As the general procedure was the same and only the amount of chloroform added to the vaccine virus was varied the details of the experiment will not be gone into.

Experiment 5.—In this test 0.4 cc. of chloroform was used. Injection of as much as 2 cc. failed to produce a lesion and attempts to reactivate with "culture" fluid and kieselguhr failed to give results. The two animals developed no immunity as the result of this injection, as shown by the fact that they had an eruption 2 months later when inoculated with untreated virus.

Experiment 6.—When 0.25 cc. of chloroform was used all the injections were negative and the two animals showed no immunity on subsequent injection with fresh virus.

Experiment 7.—In this test 0.2 cc. of chloroform was used. Injection of 4 cc. of the vaccine after treatment failed to elicit a response in one rabbit and there was no reactivation by the auxiliary agent in another and no immunity developed as the result of the injection.

Experiment 8.—The above experiment with 0.2 cc. of chloroform was repeated. This time there was a slight positive eruption following the injection of chloroformed vaccine virus plus the fluid from embryonic tissue "cultures." There was also a positive result from the injection of 4.5 cc. of the treated virus alone and slight positiveness with 1 cc. and 0.5 cc. but the lesion from the latter developed some 3 or 4 days later than the lesions from the reactivated virus. All of these animals proved immune on subsequent inoculation with fresh virus.

Experiment 9.—With 0.15 cc. of chloroform the mixture of 0.5 cc. each of the treated vaccine and the supernatant fluid of the embryonic tissue "culture" gave typical eruptions. A large amount of this virus, 4.5 cc., also gave a positive result alone but smaller amounts, 0.5 to 1 cc., resulted in very slight responses. All the animals were immune on second inoculation.

Experiment 10.—Another experiment using 0.15 cc. of chloroform was carried out. In the tests the chloroformed vaccine alone even in the smaller doses gave typical eruptions. Very little difference could be made out between these and the eruptions caused by the virus plus the auxiliary fluids. All the animals were immune on second inoculation with untreated virus.

The results seem comparable to those obtained by Flu in his study of the effect of chloroform on the agent of the chicken tumor. Where no lesions result from the injection of large amounts of the treated vaccine virus it proved impossible to reactivate the smaller doses with the fluid from embryo tissue "cultures" and no immunity is developed by the animal. Our experiments show further the great variability in the eruption produced by the same dose of vaccine virus especially when one at the lower effective limit is employed. Not only do individuals differ in susceptibility but areas in the same animal differ in their response to the same dose. An extreme example of this is to be seen in the case of Rabbit 2 of Experiment 4, in which 5 cc. of the chloroformed virus failed to produce a lesion while a typical reaction occurred in another skin area receiving only 0.5 cc. While it is conceivable that errors in technique, local injury from shaving, leakage of the fluid from the puncture wound or some such factor might explain these inconsistencies, yet as far as our observations go they exist.

Localizing Effect of Auxiliary Agents.

An occasional animal of the preliminary experiments having pronounced lesions from pure vaccine showed a typical vaccinal eruption in spots injected only with fluid from an embryonic tissue "culture." This suggested a test of the secondary fluids as localizing agents when the virus was given intravenously. It will be recalled that Calmette and Guerin (13) found that the pulling out of the hair was sufficient to localize the vaccine lesion.

Experiment.—Embryonic tissue "cultures," chicken sarcoma "cultures" and light suspensions of kieselguhr were prepared in the manner already described. As a fourth substance a 10 per cent solution of peptone was injected into the skin in 0.5 to 1 cc. amounts. The supernatant fluid from the "cultures" and the kieselguhr were used in the same amounts. Five cc. of a 10 per cent suspension of vaccine virus was injected into the ear vein of the rabbits. The animals had been shaved carefully so as to avoid injury to the skin, prior to the intradermal injection of the fluids. The resultant eruptions were tested by removing the skin area, grinding and injecting into the skin of a fresh animal, and only these eruptions proving to have active virus by this test were included as positives.

In the experiment proper eight animals were injected in five or six different spots with the fluids prepared as described above, followed by the intravenous injection of the vaccine virus. In addition seven rabbits were used to test the presence of active virus in the resultant lesions. The results are given in Table V.

TABLE V.

	Spots injected	Positive localization	Doubtful localization	Negative localization	
Embryonic "cultures"	29	25	1	3	
Chicken sarcoma "cultures"	9	6	—	3	The 3 negative cases belong to a rabbit injected with a very clear supernatant fluid
Peptone	4	—		4	
Kieselguhr	6	—	—	6	

The above results suggest that the action of the secondary fluid is not on the virus but upon the cells rendering them more susceptible to its action. While the number of tests with kieselguhr is small the

failure of the virus to localize in a single instance in the area of reaction produced by this agent is significant. It is of interest in this connection to note that the agent of the chicken tumor on intravenous injection shows less tendency to localize in kieselguhr reactions than in the reactions produced by the several other substances (14).

DISCUSSION.

It was the purpose of these experiments to parallel the work of Gye, but with vaccine virus instead of the chicken tumor agent. In planning of the tests, we took into consideration the critical work of Murphy and of Flu as well as the various results obtained by several other investigators who have attempted to repeat Gye's experiments. While the vaccine virus has certain advantages in a study of this kind, the chicken tumor agent has a special one in that natural resistance against it is so feeble that a tumor once started rarely fails to progress. In spite of the variability in the reactions reported in this paper, sufficient data of a positive nature are provided to justify certain conclusions.

Flu states that an auxiliary substance will render a small amount of the chloroformed chicken tumor filtrate infective only when a large dose of the chloroformed filtrate alone is capable of inducing a tumor. The evidence brought out by the present experiments indicates that the same is true of the vaccine virus. When the amount of chloroform used was large enough to render even great amounts of the virus innocuous no reactivation of the small dose proved possible. It is of interest to note that about the same amount of chloroform is required to inactivate the virus and the tumor agent. It would seem from these results that chloroform in amounts which still allow reactivation of the virus does not destroy all of the agent. One may suppose either that the number of infective elements is greatly reduced in number or that the infectivity of all of the elements is lowered by the chloroform treatment.

The secondary substances or activators would seem to exert their effect by rendering the animal's cells more susceptible to the infecting power of the virus. This is indicated by the result of the experiments in which it was demonstrated that the fluids most effective in reac-

tivating the chloroformed virus will induce a reaction in the skin that is favorable to localization of virus injected intravenously. The fact that some substances are more active than others in this respect regardless of the amount of injury induced, opens up an interesting question as to the type of injury or reaction which determines the localization of an infective agent. This point would bear further investigation not only as concerns the vaccine virus but the chicken tumor agent as well.

The analogy between these results and those obtained by Gye with the chicken tumor agent would tend to uphold the conclusions of Murphy and of Flu that the chloroform treatment attenuates the agent but does not destroy it; and the secondary factor contained in the "cultures" is non-specific in its action, merely rendering the cells of the inoculated animal more susceptible to the enfeebled agent.

Summary and Conclusions.

Vaccine virus, obtained from testicular inoculation shows a high susceptibility to chloroform as compared with ether, toluene, 95 per cent alcohol and acetone.

Vaccine virus, after treatment with an amount of chloroform sufficient to render it incapable or only barely capable of originating an eruption in the rabbit's skin, produces a characteristic eruption when injected with the supernatant fluid of embryonic tissue or sarcoma tissue "cultures" or kieselguhr, substances all of which are markedly irritative to the rabbit's skin.

Reactivation of the chloroformed vaccine virus is not possible when chloroform has been added to it in such quantity that the injection of large amounts of the treated virus fails to cause an eruption. Whenever reactivation has been accomplished it has been possible to get a vaccine eruption of greater or less intensity by the injection of large amounts of the chloroformed vaccine alone.

Embryo and chicken sarcoma "culture" fluids when injected intradermally make the skin susceptible to the localization of the virus introduced intravenously.

The bearing of these experiments on the interpretation of Gye's theory of cancer causation is discussed.

BIBLIOGRAPHY.

1. Gye, W. E., *Lancet*, 1925, ii, 109; 1926, ii, 989; *Brit. Med. J.*, 1925, ii, 189; 1926, ii, 865.
2. Barnard, J. S., *Lancet*, 1925, ii, 117; *Brit. Med. J.*, 1925, ii, 192.
3. Mackenzie, R. D., and Ellingworth, C. F. W., *Lancet*, 1926, ii, 745.
4. Baker, S. L., *Brit. J. Exp. Path.*, 1926, vii, 377.
5. Haskins, M. J., Schamberg, J. F., Kolmer, J. A., and Kast, C. M., *J. Cancer Research*, 1926, x, 66.
6. Simon, C. E., and Beck, M. D., *Am. J. Hyg.*, 1926, vi, 659.
7. Pentimalli, F., *Sperimentale, Fasc.*, 1926, v-vi, 623.
8. Mueller, H., *J. Exp. Med.*, 1927, xlv, 243.
9. Murphy, Jas. B., *J. Am. Med. Assn.*, 1926, lxxxvi, 1270.
10. Flu, P. C., *Compt. rend. Soc. biol.*, 1926, xciv, 1315.
11. Cori, C. F., *Proc. Soc. Exp. Biol. and Med.*, 1926, xxiv, 65.
12. Harde, E., *J. Trop. Med. and Hyg.*, 1926, xxix, 159; Romme, M., and Harde, E., *Compt. rend. Soc. biol.*, 1925, xcii, 1263.
13. Calmette and Guerin, *Ann. Inst. Pasteur*, 1901, xv, 161.
14. Mackenzie, R. D., and Sturm, E., *J. Exp. Med.*, 1928, xlvii, 345.

A STUDY OF THE MECHANISM OF NUCLEINATE-INDUCED LEUCOPENIC AND LEUCOCYTIC STATES, WITH SPECIAL REFERENCE TO THE RELATIVE RÔLES OF LIVER, SPLEEN, AND BONE MARROW.

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Plates 21 and 22.

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The newer concept that there is normally a wide range of fluctuation in the total number of circulating red and white cells (1–5), reopens the whole question of the meaning of variations in blood counts. To many conditions, such as digestion and exercise, and to many chemical substances have been attributed specific changes in the blood count, which now are known to fall within the more recently established limits of the normal. Hence, in distinguishing between the delivery of new cells from marrow or lymph glands, and changes in distribution of cells already within the circulation, it becomes necessary to study all the factors that might be involved. Each particular stimulus under consideration must be known with reference to its particular point and mode of activity. As will be demonstrated in this paper the subject can only be analyzed in the living animal, for the position of cells in the blood vessels after death is no indication of their distribution during life. In the problem it is essential to study the influence of vasomotor reactions on the distribution of blood cells, to consider possible changes in blood volume, and especially to analyze the rôle of various organs on the peripheral concentration of the cells.

The observation by Sabin (6), in a survey of normal living blood cells, of certain "non-motile" polymorphonuclear neutrophils, and the

later determination (1), of their occurrence in showers in the peripheral blood, led to the hypothesis that the effective normal stimulus might be the liberated products from disintegrating cells. This could occur either directly or through the medium of the phagocytic group of cells reducing the debris. The correlation of the showers of "nonmotile" cells in human pathological conditions with a subsequent increase of young motile neutrophils (7) further supported the concept. This seemed to point to a thorough restudy of the effect of nucleic acid and its derivatives on the leucopoietic system, inasmuch as these substances must be constantly produced physiologically in the body.

Nucleic acid was first isolated by Altmann (1877) from Miescher's nuclein. Miescher worked chiefly with pus, so readily obtained in those days of septic surgery, and since pus consisted mostly of nuclei, and his minimal residue was cytoplasmic, he reasoned that the material he obtained, which was high in phosphorous content, came from the nucleus, and hence the name "nuclein" (8).

Following the emphasis placed by Metchnikoff (9) on the "phagocytic power" of the polymorphonuclear leucocytes as the *modus operandi* for destroying bacteria, Ames and Huntley (10) were among the first to study nucleic acid experimentally from the standpoint of its apparent production of a leucocytosis. They concluded from the hypodermic injection of nuclein solution into dogs that there was an increase in the number of leucocytes in the central and peripheral circulation, and, further, since they found an increase of young cells, that the response was a true delivery of cells from the bone marrow. Milroy and Malcolm (11), using the sodium salts of yeast and thymus nucleic acid and their decomposition products, thymic acid, adenine, guanine, and cystosine, found after subcutaneous injections in rabbits and guinea pigs what they interpreted as a specific hyperleucocytosis, but the increases were from 5500 to 6800, or from 6000 to 7500, a range of variation hardly permissible of such interpretation today.

The first advocacy of nuclein therapy was on purely theoretical grounds, the claim being made, for instance by Vaughan, Novy, and McClintock (12), that by its use the germicidal power of the blood could be increased and hence the resistance heightened to diseases of microbic origin. However, the effectiveness of nucleic acid, in certain clinical conditions, through its apparent stimulation of an increase in the white count, has been periodically suggested by both physicians and surgeons (13–18), though the increases in white cells, which have been reported, are equivocal, and, for the most part, fall within the physiological range for the white count. Thus Habetin (19), in 1923, advocated the injection of 10 cc. of a 5 per cent solution of sodium nucleinate subcutaneously as a clinical test of bone marrow function, describing the reaction in healthy individuals as an increase of from 40 to 60 per cent of cells, whereas, as is now known, the normal range of

fluctuation is 100 per cent in 24 hours (1, 2). Larsell, Jones, Nokes, and Phillips (20, 21) recently reported an experimental and clinical study of the hemopoietic effects of intravenously injected nucleic acids. In rabbits they found hemopoiesis stimulated by the nucleic acid from the nuclei of bird's cells, and in selected human cases of anemia they describe a comparable but temporary hemopoietic stimulation. Robertson, Hicks, and Marston (22), on the other hand, found no leucocytosis, either relative or absolute, in human subjects on an otherwise purine-free diet, following the oral ingestion of nucleic acids of vegetable or animal origin.

Löwit (23) ascribed the "leucopenia" he found following injections of peptone, nucleins, and sodium urate to a leucolytic phenomenon in the inner organs; the leucocytosis which followed he thought a function of this lysis reflected in a direct response from the bone marrow. These leucopenic-leucocytic fluctuations of the white cells have been the subject of study and conjecture ever since, the mechanism having been in turn attributed to lysis (23), redistribution phenomena (24, 25, 26), positive and negative chemotaxis either within the circulation only (27) or between circulation and marrow.

That additional data relative to the fundamental mechanism of the changes ascribed to nucleinate might be available, the series of experimental observations here reported were begun in the fall of 1924.

Technical Detail.

Sodium nucleinate (Merck) from yeast nucleic acid was made up in fresh, glass-distilled water so that each cc. contained 100 mg. There was no essential difference in the action of two different lots from this source nor in a sample obtained from Dr. Henry Jackson, Jr. The biuret reaction for protein was negative when the test was applied to the sodium nucleinate of these experiments. The adenine and guanine nucleotides first used were prepared by the autoclave method of Jones (28) and made available also through the kindness of Dr. Jackson of the Thorndike Laboratory. Chemically pure, crystalline adenylic and guanylic acids were later supplied through the generosity of Dr. P. A. Levene of The Rockefeller Institute, and used with results entirely comparable in every respect with those obtained from the nucleotide products from the first source. The solutions before injection were brought to pH 7.8 by the addition of N/10 sodium hydroxide. A relatively large dosage was employed, in the majority of instances 1 gm. in 10 cc., in order that the effects, if any, might be decided enough for detection and analysis of the mechanism. Clinically there was considerable variation in the severity of the reaction in rabbits to the intravenous injections of 1 gm. of the sodium nucleinate, a more or less marked vasoconstriction occurring for from 15 minutes to ½ hour, followed by vasodilatation and transitory diarrhea; the temperature registered at times 105°F. (normal for rabbits 102–104°). The injection of 2 gm. of sodium nucleinate intravenously in Cat 1, respiratory and myocardiographic tracings be-

ing recorded on the kymograph, showed a very transitory increase in the rate, but not in the amplitude of the respirations, and a moderate rise in the blood pressure maintained for a somewhat longer period. As the nucleotides are less soluble than the nucleinate in distilled water and tend to form a gel if the proper pH is not maintained, care is necessary in their preparation for intravenous use. However, given in the proper dilutions the guanine and adenine nucleotides did not produce the clinical symptoms of toxicity and fever noted with sodium nucleinate in certain animals.

All counts were made with Bureau of Standards equipment. The hemoglobins were read in a Duboscq colorimeter with the Newcomer standard. The hematocrit determinations were made with the Van Allen pipette. Refractometric readings were made for the serum protein estimations. For the Arneth differential counts cover-slip preparations were stained with Wright's blood stain and 100 cells counted from each slip and the average percentages taken. In some of the experiments the differential counts of the white cells were made with the supravital technic (6) using vital neutral red. The usual routine was employed in the taking of the various consecutive samples (7).

In the experiments in which comparable observations were made from ear vein, heart, and liver, the preparations were taken by three people simultaneously. Through the anterior mediastinum a needle was introduced into the right ventricle; at the same time a small triangle of liver tissue was removed through a small abdominal incision and fixed at once in Helly's fluid for histological study, the count being taken from the blood welling forth from the freshly cut surface. A warm sponge controlled the hemorrhage from the liver, and each succeeding sample was taken from a fresh area far enough removed (2 cm.) so that the former manipulation could not affect the local concentration of white cells. Similar consecutive samples of the spleen were taken for fixation and studied histologically in series, total counts from the splenic pulp being impossible for comparative studies. All such experiments were performed under general anesthesia with sodium-barbital, 0.4 gm. per kilo being injected intravenously prior to operation. Repeated control counts from the peripheral blood were taken before and after the sodium-barbital and during the operative procedures, in order to establish their influence on the cellular equilibrium of the individual animal before any special substances were introduced.

The rabbits undergoing splenectomy were operated upon with aseptic surgical technic, under ether anesthesia, and peripheral blood counts as well as other clinical observations were used as an index of their condition before special procedures were undertaken. In only one instance was appreciable leucocytosis noted following, and apparently related to, splenectomy.

Through the courtesy of Dr. Joseph T. Wearn, it was possible to carry out experiments with the oncometer in which changes in the volume of the spleen, during the reaction to sodium nucleinate, were recorded on the kymograph. Because of the small calibre of the vessels entering and leaving the spleen in the rabbit, it is difficult to secure excursions of systole and diastole, such, for example,

as are observed with the kidney; nevertheless, the pulsations of the arteries entering the spleen were plainly visible through the glass oncometer at all times, the return flow through the veins was unobstructed, a constant temperature was maintained, and the tracings recorded were in every particular identical with those reproduced by Roy (29), Schäfer and Moore (30), and others. Therefore, it is believed that these observations may be taken as at least tending to support the general conclusions with reference to the spleen, that have been reached by the other avenues of approach to the problem.

Forty-one rabbits were studied. Many of them were used for repeated experiments for comparison and contrast of the same and different substances in the same individuals, as may be seen from the charts. Six experiments were performed, in which a survey of peripheral blood, heart, and liver after sodium nucleinate was made every 15 minutes, repeated samples of liver and spleen being removed for histological study. Uncomplicated studies of the peripheral blood after sodium nucleinate were made in six rabbits, after guanine nucleotide in six rabbits, and after adenine nucleotide in three. Splenectomy was performed in seven rabbits; and tracings with the spleen in the oncometer were taken in ten animals to correlate with cellular data, from the peripheral blood, after sodium nucleinate. Correlation of repeated differential counts from peripheral blood and splenic puncture, taken before and after sodium nucleinate, was made in two animals (Table I).

The Bone Marrow during the Reaction to Sodium Nucleinate.

Following the intravenous administration of 1 gm. of sodium nucleinate into normal rabbits, there developed immediately in practically every instance a leucopenia which lasted for several hours (Charts 1 to 3 A). No leucolytic effect could be observed in *in vitro* tests made according to a method previously described (31) and the transitory vasomotor disturbances in the peripheral vessels passed off much sooner than the leucopenia. A leucocytosis, usually of considerable magnitude (50,000 to 100,000), but varying with the individual, succeeded this leucopenic period and in some instances lasted into the 2nd, 3rd, and 4th days (Chart 1). During the leucopenia the fall in total number of cells was primarily due to a decrease in the neutrophilic group (Chart 3), while the leucocytosis reflected greatly increased numbers of this cell type. The basophilic and eosinophilic leucocytes followed the fluctuations of the neutrophilic cells in miniature, disappearing entirely in the leucopenic phase and showing a slight increase in absolute number during the leucocytosis. The lymphocytes, in our experience, may or may not decrease in

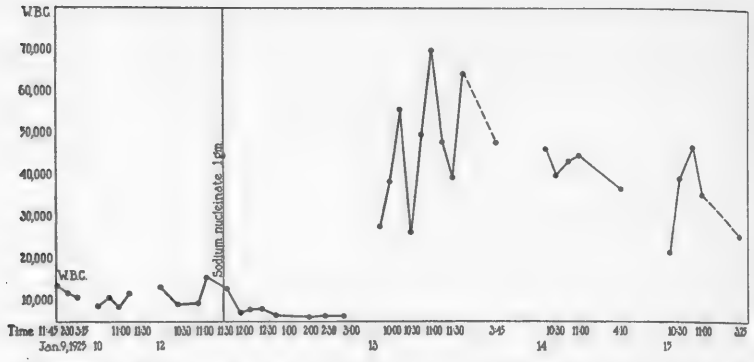


CHART 1. Rabbit 1, T 28. Leucopenia (minimum 2280) induced by 1 gm. of sodium nucleinate, and followed by a leucocytosis (maximum 69,700) on the 2nd, 3rd, and 4th days thereafter.

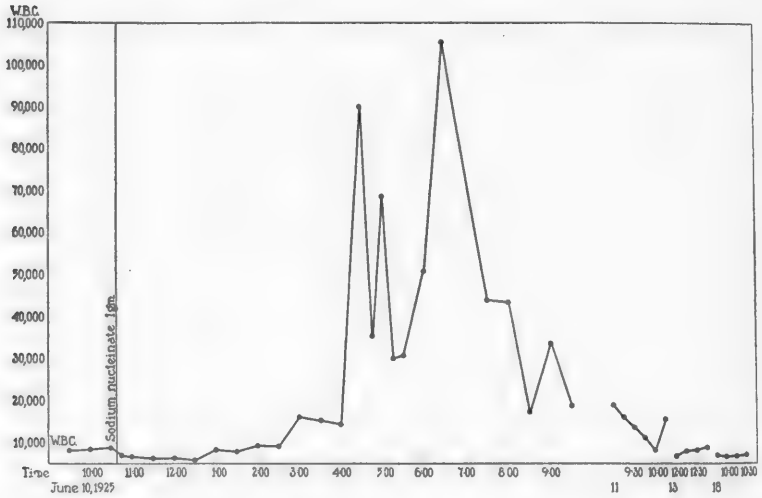


CHART 2. Rabbit 2, T 80. Leucopenia (minimum 1900) after sodium nucleinate which lasted for about 5 hours, with a leucocytosis reaching a maximum of 105,100 8 hours following the injection.

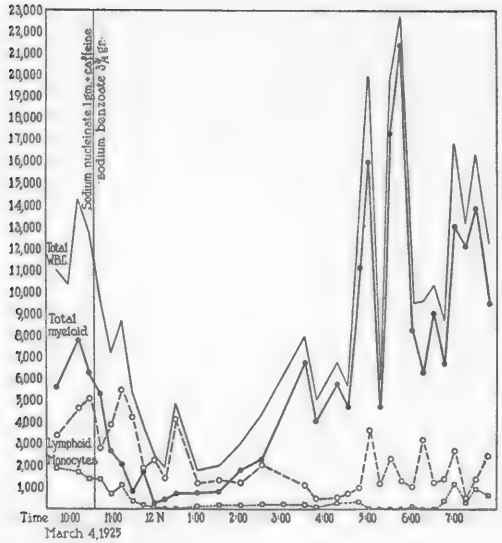


CHART 3A. Rabbit 3, T 35. There is here a depression of lymphocytes and monocytes following sodium nucleinate, but with only a return to normal thereafter, and some animals have not shown the depression in these groups. Both leucopenia and leucocytosis are primarily a reflection of variations in the myeloid group of cells originating in the bone marrow. Differential count of white cells made from cover slip films stained with Wright's blood stain.

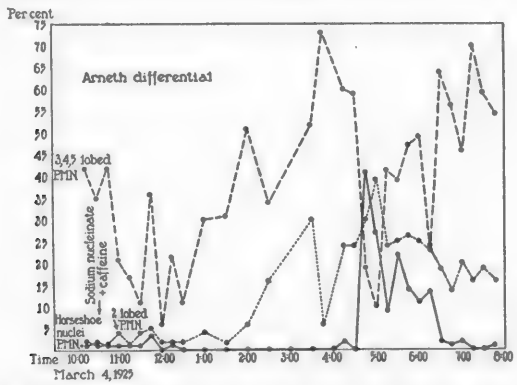


CHART 3B. Rabbit 3, T 35. There is a "shift to the left" in the Arneth pattern first noted 4 hours after sodium nucleinate and coincident with the onset of leucocytosis. This "shift" is indicative of the large part played by the bone marrow in the reaction.

absolute number during the leucopenia, but we have never observed a subsequent lymphocytosis of any moment in this series. The same may be said of the monocytes. A "shift to the left" in the Arneth pattern (Charts 3B, 10B) of the nuclei in the polymorphonuclear neutrophilic leucocytes indicated an increased delivery of cells from the bone marrow as at least one factor of importance in the phenomenon. Histological examination of the bone marrow during the hours just following the sodium nucleinate showed a markedly speeded up process of maturation of Myelocytes "C" (32, 33) into motile leucocytes; a mobilization of these cells about patent sinuses together with active diapedesis characterized the marrow in every instance.

Fig. 1 is a photomicrograph of the marrow from Rabbit 6 (Chart 6) 2 hours after the injection of 1 gm. of sodium nucleinate, while the period of leucopenia still existed in the general circulation, as indicated by counts from liver, right ventricle, and periphery. The sinus is surrounded by a continuous border of encroaching neutrophilic leucocytes in active diapedesis, the open spaces to right and left indicating the gradual depletion of the myeloid reserve of that focus. This appearance is identical with that observed after the injection of inactivated typhoid organisms (Figs. 22 to 24 in a former paper (34)). Fig. 2 shows a marrow after two injections of sodium nucleinate, the second having been given on the 4th day following the first, when the count had regained the normal level. The depletion of the myeloid elements with the beginning displacement of the fat cells preliminary to reparative metaplasia reveals a latent response on the part of the marrow, similar to that when a demand is made for increased cells from the late myelocyte level without the accompanying presence of a maturation factor (33, 35).

Analysis seemed to indicate that the response from the bone marrow to nucleinate preceded the subsidence of the leucopenia. To measure the response and to find if possible the location of the white cells during the leucopenic phase, surveys of certain of the abdominal viscera were undertaken.

The Liver and the Lungs during the Reaction to Sodium Nucleinate.

In none of the experiments was there observed a rise in the cells in the liver during a fall in the peripheral blood, while the circulation of the animal was maintained unimpaired.

Charts 4 to 6 give the concentration of white cells in ear vein, right heart, and liver with the animal under sodium-barbital anesthesia and the abdomen opened

under aseptic precautions. In each instance the leucopenia following sodium nucleinate was registered in the right ventricle and liver as well as in the peripheral blood, with no gross evidence of dilatation of the vessels of the splanchnic area. In both Rabbits 5 (Chart 5) and 6 (Chart 6) the counts in the peripheral blood

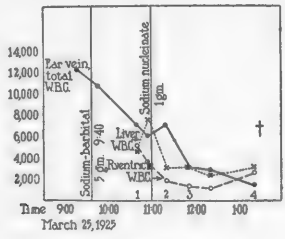


CHART 4.

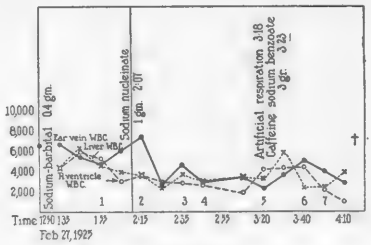


CHART 5.

CHART 4. Rabbit 4, T 49. The leucopenia following sodium nucleinate is the expression of a depression in the total white cells in the general circulation (counts from peripheral vein, right ventricle, and liver). The situation of the digits along the abscissa in Charts 4, 5, and 6 indicates the points at which liver and spleen tissue were removed for histological study.

CHART 5. Rabbit 5, T 37. The changes in total white cells following sodium nucleinate, the institution of artificial respiration, and the administration of caffeine are similar; specimens from periphery, right ventricle, and liver.

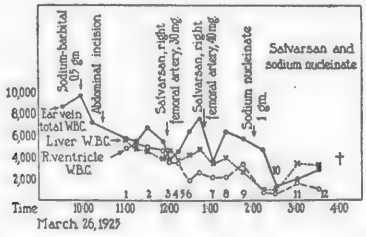


CHART 6. Rabbit 6, T 50. The leucopenia after salvarsan or sodium nucleinate occurs both in the central and peripheral blood; successive simultaneous counts from ear vein, right heart, and liver.

were higher, not lower than the other counts taken simultaneously, but the general trend of the total counts was in the same direction. Two injections of salvarsan in Rabbit 6 gave temporary depressions in the total cells without an increase in cells in the liver, though the transitory leucopenia following such injections has been attributed to this organ (36). The figures along the abscissa in each chart

indicate the points at which tissues from spleen and liver were removed for fixation. The microscopic study of sections of the liver from all the animals of the series showed that there had been no accumulation of leucocytes in the capillary bed of the organ during leucopenia.

Most of the estimations of the local concentration of cells, heretofore, have been made from histological sections of postmortem material or from counts from the liver after the circulation had ceased. In following successive counts from the liver both before and after death the explanation may be seen, perhaps, for the conception, generally current, of the large part played by the liver as a storehouse for the white cells in leucopenia. The profound disturbance in distribution of cells in the circulation at the moment of death makes it essential to take all preparations for total and differential counts of blood cells from organs while the animal is still alive and in good condition, that is to say, not in a state of shock.

In Chart 7 it will be seen that during the leucopenia the white cells in the liver remained low and only began to rise gradually coincident with the rise of cells in the general circulation. However, within 2 minutes of the death of the animal, the total count of white cells in the liver had risen from 11,000 to 37,000; the differential counts of the white cells, on the other hand, at 4:20 and at 4:24 were identical, a finding quite at variance with the increase as analyzed in the peripheral blood during the leucocytosis following sodium nucleinate (Chart 3). In this animal red cell counts were taken from periphery and liver at the same time that the white counts were being noted, in order that any changes in blood volume might be indirectly estimated. The remarkable constancy of the variations in the number of red cells in liver and periphery shows the specificity of the fluctuations in white blood cells.

Rabbit 8 (T 67) was splenectomized. The counts in liver and periphery after sodium nucleinate were 5200 and 8800 respectively. During the experiment the animal died and 2 minutes after death the white cells in the liver were 23,700 per c.mm. In this instance also red cells from liver and ear vein were taken, as in Rabbit 7, and showed the same gradual fall in total numbers per cc. In Rabbit 15 (T 101), uninjected and normal, the total count from the liver just prior to death by air embolism was 6300, 1 minute after death it was 10,300, and 6 minutes after, 19,000. Rabbit 16 (T 91) showed 8000 cells in the liver just before death by air embolism, at the end of an experiment with the spleen in an oncometer during sodium nucleinate leucopenia and leucocytosis, while 2 minutes after death the total white cells in the same organ were 24,000 per c.mm.

Thus, whether under normal conditions, or in the splenectomized animal, whether during the height of the leucopenia or during the

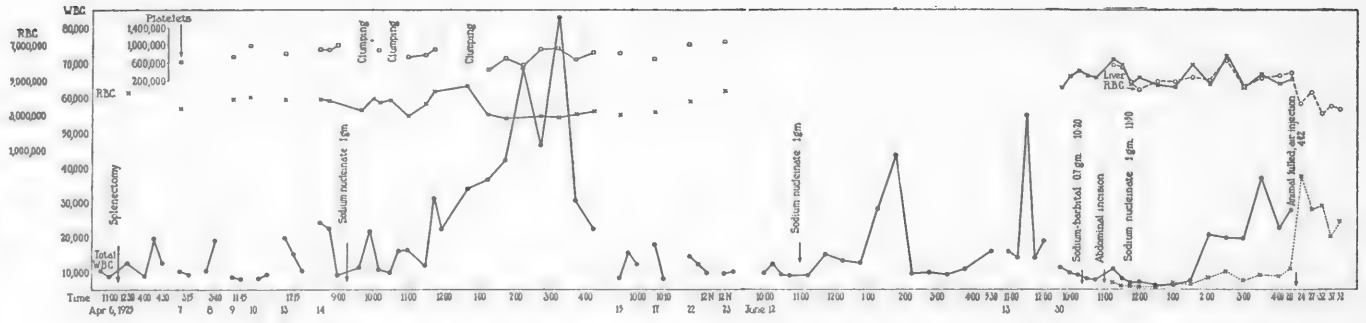


CHART 7. Rabbit 7, T 54. On three successive occasions over a period of 3 months leucocytosis followed within 2½ hours the injection of 1 gm. of sodium nucleinate into the splenectomized rabbit. Platelets and red cells are charted for comparison. On the last occasion red and white cells from liver and periphery were counted and the experiment terminated by air embolism. The immediate change in the concentration of the white cells in the liver after cessation of the circulation is graphically recorded.

leucocytosis after sodium nucleinate, consecutive counts taken from the liver before and after death show the same marked relative discrepancy in total numbers, though not in differential counts. In other words, the sudden increase in total white cells in the liver immediately after death is due to a redistribution of the white cells in the circulation at the moment of death, and not to a sudden influx of new cells from the bone marrow or spleen, as when a true increase in available cells of any type occurs. It would seem, therefore, that all estimates of redistribution phenomena must be made with the circulation unimpaired, and that, under the conditions of the experiments here cited, the changes within the liver blood do not initiate but reflect the leucocytosis following leucopenia after sodium nucleinate and salvarsan. There is within the organ no concentration of cells during the period of depression other than that found in the general circulation. All sections studied in this series showing increased numbers of granulocytes in the capillaries of the liver were taken at a time when there was a leucocytosis in the peripheral blood also. All tissue was taken from the anesthetized living animal and fixed immediately.

Since these observations were made, A. F. B. Shaw (2) and Webb (37) have reported similar studies on leucocytic distribution. Shaw found in normal rabbits and dogs under chloretone a uniform distribution of leucocytes at any given moment throughout the body, *viz.*, periphery, brain, liver, kidney, lung, mesenteric vein, and both sides of the heart. He emphasized the dangers in prolonged experiments of maldistribution of the leucocytes, induced, presumably, by circulatory disturbances following shock, and reported profound inequality in body distribution of leucocytes immediately after death. The problem of leucocytic distribution in the dog's body during horse serum anaphylaxis was studied by Webb, who found the transitory decrease in total cells from the capillary blood of liver, intestines, kidney, spleen, bone marrow, and from portal vein to parallel that of the periphery. This was controlled by the study of sections which revealed no aggregations of leucocytes within the capillaries of these organs. However, from the pulmonary vein during acute anaphylactic shock he obtained fewer leucocytes than from the pulmonary artery, and sections showed enormous numbers of leucocytes in the capillaries of the lungs. This confirms the earlier findings for the lung of Goldscheider and Jacob (24), Bruce (38), Andrewes (39), and others working with protein and bacterial antigens. However, Schilling (40), in studying Oeller's (41) tissues after anaphylaxis, analyzed this finding of specific accumulations of leucocytes in capillaries of the lung as secondary to the enormous local swelling of the endothelial cells. This would seem, therefore, to be a special

phenomenon associated with anaphylactoid sensitivity, the leucopenia being secondary to the mechanical factors involved in local swelling of the endothelium of the pulmonary capillaries. On the other hand, a study of the lungs during the leucopenic phase after sodium nucleinate, or as Wells (42) found after bacterial injections, showed no such accumulations of leucocytes to account for the peripheral leucopenia. Wells studied the leucopenia in rabbits following bacterial injections, and reported the finding of uniformly low counts from the blood of splenic and mesenteric arteries, splenic, portal, hepatic, and superior mesenteric veins, the parenchyma of the lung, both ventricles of the heart, and the marginal ear vein. In sharp contrast were the number of leucocytes obtained from bone marrow and liver, and especially from the blood of the parenchyma of the spleen.

TABLE I.

Relationship of Myeloid Cells in Peripheral Blood and Spleen Following Sodium Nucleinate.

Rabbit No.	Time	W.B.C. peripheral blood				Spleen puncture			
		Total	Myeloid	Lymphoid	Monocytes	Myeloid	Lymphoid	Monocytes	Clasmatocytes
17	Before Na nucleinate	7000	66	24	10	19	78	2	1
T98	1¼ hrs. after Na nucleinate	6000	47	45	8	40	56	4	0
18	Before Na nucleinate	18000	64	31	5	16	78	1.5	4.5
T99	1 hr. after Na nucleinate	8400	30	68	2	30	66	2	2
	2¼ hrs. after Na nucleinate	35000	82	14	4	48	48	1	3

The Spleen during the Reaction to Sodium Nucleinate.

Having demonstrated that the leucopenia was not due to a stasis of leucocytes in the liver, lungs, or splanchnic area we turned more directly to the spleen as the organ of all the abdominal viscera most likely to be involved in the general phenomena in which we were interested, both from the nature of its anatomical structure and from what is known of its function. Because of the large number of cells in the spleen not in circulation at any given moment, total counts to compare with those from heart and liver were not attempted, but instead the differential count of cells, obtained by puncture with a fine capillary pipette, was made with the supravital technic (43), and compared with the findings in the peripheral blood. With this method

successive splenic punctures during the leucopenic-leucocytic periods following sodium nucleinate showed a gradual but definite increase in percentage of neutrophils in the splenic parenchyma.

It will be seen from Table I that the myeloid cells may be considered to make up from 15 to 20 per cent of the cells obtained under the conditions of splenic puncture. In Rabbit 17 (T 98), 1¼ hours after sodium nucleinate, they had increased 21 per cent, or to 40 per cent of all the cells, while the myeloid cells in the peripheral blood had decreased from 66 to 47 per cent. In Rabbit 18 (T 99) the increase of myeloid cells in the spleen was from 16 to 30 per cent in 1 hour after sodium nucleinate, and to 48 per cent in 2¼ hours, while the peripheral count fell from 18,000 to 8400 and myeloid cells from 64 to 30 per cent the 1st hour. The total number of lymphocytes in the peripheral blood throughout remained relatively constant though showing percentage fluctuations of 31 to 68 to 14 per cent, again emphasizing the specificity of the effect of the nucleinate on the neutrophilic group of white cells.

The histological evidence from the fixed sections of spleen, removed in series at the times when the specimens of the liver, previously mentioned, were taken, shows a gradual increase in neutrophilic leucocytes in the parenchyma of the spleen following sodium nucleinate.

In Charts 4 to 6 the points at which splenic tissue was removed for section are indicated by the digits along the abscissæ. Fig. 3 represents the normal spleen of Rabbit 4. Fig. 4 shows another portion of the same spleen 1 hour after sodium nucleinate (see Points 1 and 3 as marked on Chart 4). The beginning accumulation of small foci of granulocytes in Fig. 4, more particularly, at this early stage after the nucleinate, near the vessels, may be seen. The diffuse scattering of increased numbers of individual neutrophils throughout the parenchyma of the organ is not so apparent at this magnification. Fig. 5 is a photomicrograph of a section of the spleen of Rabbit 19 (T 31), 8 hours after sodium nucleinate during a leucocytosis reaching 60,000 to 70,000, which had started 3 hours before, after a leucopenic period of 5 hours.

This is representative of several experiments tending to show that the leucocytosis following the leucopenia is not simply the releasing of the cells stored up during the leucopenic period, in other words a temporary redistribution phenomenon, but the result instead of an increased activity of the hemopoietic centers, initiated early and maintained for considerable periods.

The series of oncometer experiments for the study of changes in volume of the spleen during the leucopenia-leucocytosis reaction to sodium nucleinate reveal a definite increase in the size of this organ.

The kymograph records, covering periods of hours, are far too voluminous to reproduce except in very schematic form, but in all particulars they resemble those illustrated in similar studies on the spleen (29, 30). The relative changes in splenic volume, as recorded and synchronized with the total white count from the peripheral blood, are represented diagrammatically in Chart 15. The control period gave the well recognized inherent contractions of the spleen during the base line observations 45 minutes before 1 gm. of sodium nucleinate was injected into the ear vein. Coincident with the onset of leucopenia, the splenic volume started to increase; when the count first began to rise (5.25 p.m.), there was a slight contraction of the spleen, but as the count reached 50,000, the volume again increased, reaching an equilibrium. At a moment when the total count had fallen again to about 10,000, 4 cc. of a 1:1000 solution of adrenalin was introduced into the vein; this was followed immediately by a strong contraction of the spleen and a coincident rise in the white count to 26,400; after the return to the former volume and white count, mechanical stimulation of the saphenous nerve gave a similar synchronous fall in spleen volume and rise in peripheral white count, differing only quantitatively from that previously recorded.

Roy (29), who first adapted the plethysmograph to the investigation of changes in organ volume, described the rhythmic contractions of the normal spleen in dog, cat, and rabbit, and found that stimulation of the central end of a cut sensory nerve caused a rapid contraction of the spleen. Oliver and Schäfer (44) and Schäfer and Moore (30) confirmed this and described an enormous contraction of the spleen after the injection of extracts of the suprarenal gland.

Apparently in a state when the spleen is known to contain large numbers of myeloid cells, as illustrated in the case of Rabbit 19 (Fig. 5), any marked contraction of the organ might be expected to force out neutrophils, even as it does lymphocytes, under adrenalin stimulation in the normal (7). Barcroft's (45) conclusions with reference to the relation of spleen and the reserve of red cells would seem equally apropos for the white cells under the conditions just cited: "It (the spleen) has a function entirely in conformity with its muscular structure, being in fact a reservoir of corpuscles at once fitted by its reticulum to detain them, and by its musculature to expel them when required to do so." de Boer and Carroll (46) further deduced that the material expressed from the spleen was largely corpuscles (red cells) because the CO content of the blood in the splenic vein drawn during a contraction was intermediate between that of splenic artery and pulp.

A study of the platelets in the majority of the experiments here cited (Charts 7, 9, 14) revealed a clumping into large masses quite

impossible to separate for counting, during the period represented by the leucopenic phase. In the spleen an increase in the number of granulocytes phagocytized within the clasmatocytes was noted during their increasing infiltration of the parenchyma. These observations suggest that the nucleinate molecule in some way alters temporarily the surface of these cells, and of the platelets, sufficiently to permit of their adhesion and filtration in the parenchyma of the spleen, but not within the endothelial lined capillary beds of other organs. Those sufficiently injured are taken up by the phagocytic cells so abundant in the spleen normally, the remainder being returned to the circulation at a later time. That the majority of the white cells are not permanently injured or destroyed seems clear. Also the fact that lymphocytes and monocytes remain relatively undisturbed in many of the reactions militates against the concept of a purely mechanical factor in the leucopenia.

The Effect of Splenectomy on the Leucopenia Produced by Sodium Nucleinate.

The crucial test of the importance and extent of the rôle of the spleen in the leucopenia of sodium nucleinate origin is obviously to be found in observations after splenectomy. A series of splenectomies were accordingly performed before, during, and after sodium nucleinate administration.

No depression or shock, such as that described by Larsell, Nokes, and Phillips (20), was noted in the splenectomized rabbits after nucleic acid. Chart 8 shows the leucopenia following 1 gm. of sodium nucleinate with the removal of the spleen at the time which, in the majority of the animals, proved to be about the middle of the period of depression in the peripheral count. A leucocytosis reaching 52,000 was immediately precipitated. It will be seen from Charts 7, 9, and 10 A that aseptic splenectomy does not cause such a rise in the white count *per se*.

Partial splenectomy, as performed in Rabbit 9 (Chart 9), did not prevent the usual leucopenia, but after complete removal of the remaining portion of the organ at a later operation there was not the characteristic leucopenia with sodium nucleinate in the original dosage, and the leucocytosis followed more speedily, the highest count reaching 110,000. In this instance the lowered platelet count was due to the agglutination of the platelets and not to an actual decrease in their numbers, and the red counts indicate indirectly the relative constancy of the blood volume. Rabbit 7 (Chart 7) showed the onset of leucocytosis after splenectomy

within 2½ hours of the sodium nucleinate injection on three different occasions over a period of 3 months. On all three occasions the tendency of the white count to maintain its normal rhythmic equilibrium undisturbed until the real increase was apparent from the bone marrow was clear from the preliminary counts, which may be taken as an indication that the latent period of response for the hemopoietic organs under this stimulus in this individual was between 2 and 3 hours. Here again the clumping of the platelets was found but without an accompanying leucopenia as in the normal rabbits, and the close conformity of red cells from marginal ear vein and liver indirectly controls the estimation of blood volume and stasis factors.

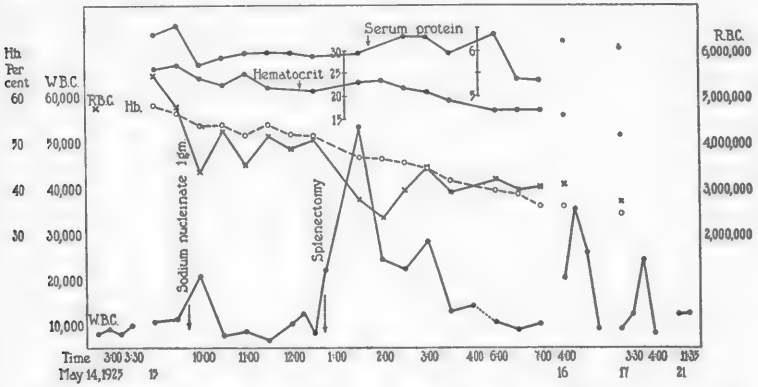


CHART 8. Rabbit 8, T 67. Splenectomy in the midst of the leucopenic period after sodium nucleinate was followed immediately by a leucocytosis reaching 55,000, a high zonal range for the white cells being maintained on the succeeding 2 days. Such a leucocytosis does not necessarily follow splenectomy, as indicated on Charts 7 and 9. Serum protein, plasma-cell ratio, hemoglobin, and total red cells are recorded as an indirect control of changes in the blood volume.

In Rabbit 10 (Chart 10A) the latent hemopoietic response to sodium nucleinate 3 days after splenectomy occurred in 1 hour and 40 minutes, and again, 18 days after, in 2½ hours, with no tendency to leucopenia comparable with that observed under identical conditions in the normal animal. Chart 10B shows the Arneth differential count of the neutrophilic leucocytes during the first experiment with the nucleinate, the "shift to the left" being apparent within an hour and marked at 2 hours. Thus the response of the bone marrow to a chemotactic stimulus, to be discussed later, was here apparent in the total count within 2 hours and qualitatively within an even shorter period. On Chart 10A in the second experiment (May 5) are recorded the complete series of observations including serum protein, cell-plasma ratio, red cells, and hemoglobin taken coincidently with the white cells

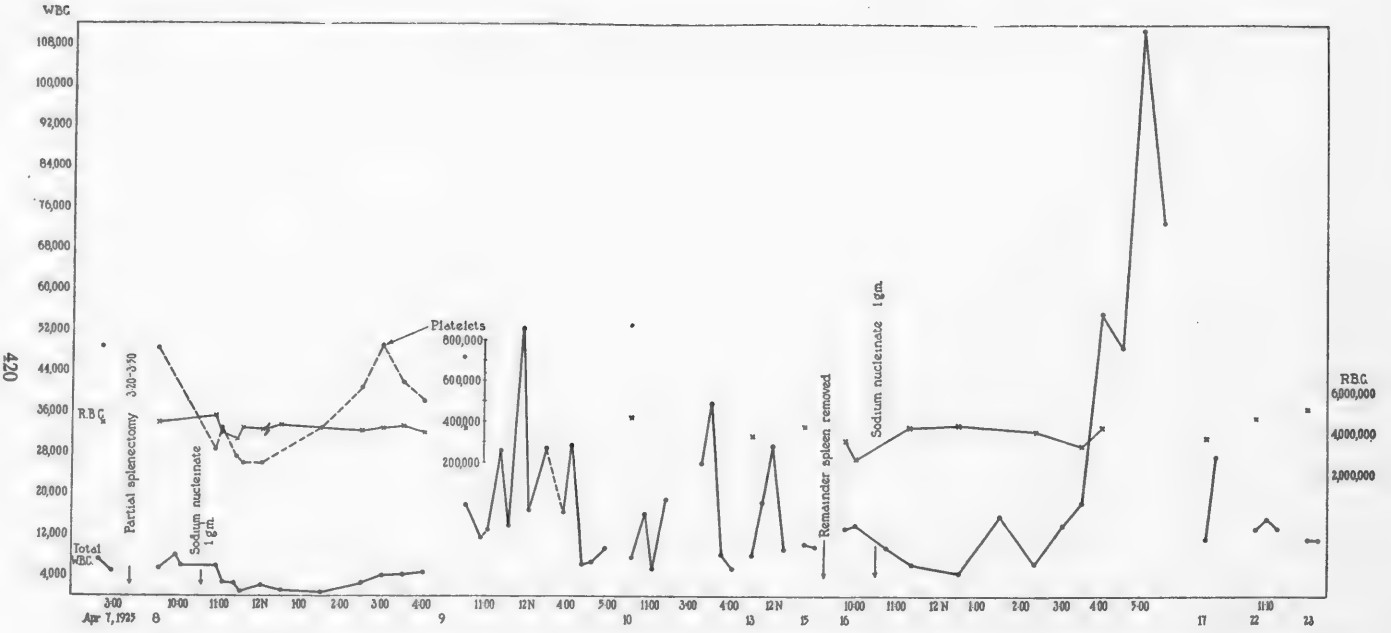


CHART 9. Rabbit 9, T 57. Partial splenectomy did not eliminate the leucopenic period of 6 hours after sodium nucleinate (minimum 1550), but removal of the remainder of the spleen at a second operation did. A total count of 111,000 was reached 6 hours after the same dosage of sodium nucleinate without the development of a comparable severe leucopenia preceding. The red cells, and the fall in platelets due to clumping, are recorded.

and indicating the specificity of the leucocytic response. In order that the condition existing during the leucocytosis so frequently observed on the day following the sodium nucleinate injections might be observed, an abdominal incision was made under sodium-barbital anesthesia, and total red and white counts taken simultaneously from liver, right ventricle, and ear vein. It will be seen that the red cells from the three sources showed comparable numbers and fluctuations, but that the white cells in the periphery showed fluctuations entirely independent of the liver, even in the absence of the spleen.

With the spleen eliminated from the rabbit sodium nucleinate, under the conditions outlined, repeatedly produced a leucocytosis, without preceding leucopenia, within a period of time one-half to one-sixth of that required in animals in which the spleen was intact, the latter invariably showing a profound leucopenia immediately following the nucleinate injection.

Scheunert and Krzywanek (47), following the work of Barcroft and his associates, have shown that for 1 year after splenectomy there is no change in erythrocyte volume after exercise, and they conclude that the spleen is the only organ which regulates the number of circulating erythrocytes. With reference to the white cells, Viale (48) has recently reported experiments with direct electric stimulation of the spleen of the dog exposed at laparotomy under chloral narcosis. The occurrence, immediately following faradic stimulation, of a leucopenia in the blood of the splenic vein with the differential count unchanged, and without changes comparable in direction or degree in the red cells, led him to ascribe the phenomenon to a contraction of the spleen with adherence of white cells to the vessel walls. Direct mechanical massage of the spleen gave no changes in red or white cells. He describes the spleen as an organ of mechanical regulation of the number of both red and white cells. Soler (49) found, on the basis of postsplenectomy leucocytosis, a capacity inherent in the spleen for selective regulation of the circulatory corpuscular elements. He proposes the term "sissoressi" from the Greek meaning "I accumulate." However, no critical analysis was made of the mechanism or cause of the leucocytosis he observed, and in our own experience aseptic splenectomy is not followed necessarily by a leucocytosis.

That the spleen may, under certain conditions, act as a temporary reservoir for myeloid white cells and thus exert something of a regulatory function, more or less beneficial, over their availability to the general circulation and tissues seems clear. As Krumbhaar (50) has pointed out in reviewing the anatomy of the spleen, "the shunts through the pulp offer splendid opportunities for storage and cellular metabolic mechanisms and in fact clinical evidence indicates that at times

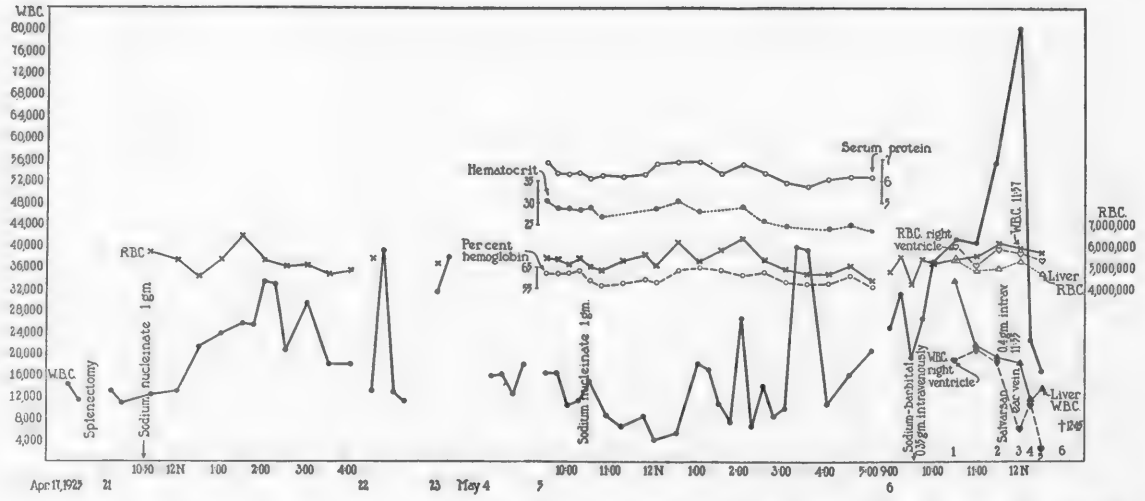


CHART 10A. Rabbit 10, T 62. Within 2 hours sodium nucleinate had given rise to a leucocytosis without preceding leucopenia in this splenectomized rabbit. Fourteen days later a similar leucocytic response was elicited within 2½ hours, the maximum counts, however, not being reached until the 2nd day. At this time successive total red and white counts were taken simultaneously from periphery, right ventricle, and liver. The counts on the red cells from the three sources run closely parallel, but those of the white cells from the peripheral vein show much greater fluctuations than those from ventricle and liver.

the backwaters thus afforded may become a positive disadvantage." The leucopenia of splenic origin after sodium nucleinate represents such a "positive disadvantage." On the other hand, it is just as clear that the spleen is not the sole regulatory mechanism for white cells in the body and that its function in this direction is accessory rather than primary. In the case of the leucopenia following injections of sodium nucleinate in the normal rabbit, the spleen seems to be the only organ involved, but that this may not be true under different conditions,

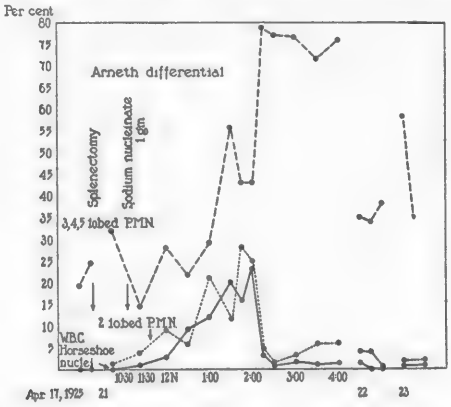


CHART 10B. Rabbit 10, T 62. In this instance the "shift to the left" in the Arneth pattern for the neutrophilic leucocytes is evident sooner than it was in the normal rabbit (see Chart 3B), and coincides with the earlier appearance of leucocytosis in the splenectomized animal. The speed of the response from the bone marrow may be deduced from the "shift" in the Arneth curve, in animals in which the spleen does not act as a reservoir for the white cells.

has been shown in the work already cited on the mechanism of leucopenia in anaphylaxis.

The Effect of Adenine and Guanine Nucleotides.

With the thought that the factor responsible for this stimulus to the neutrophilic leucocytes and apparantly acting directly on the hemopoietic tissues, might reside in one of the nucleotides of which the more complex nucleinate molecule is composed, and that the cause, mechanical or chemical, of the leucopenia might be eliminated, experiments

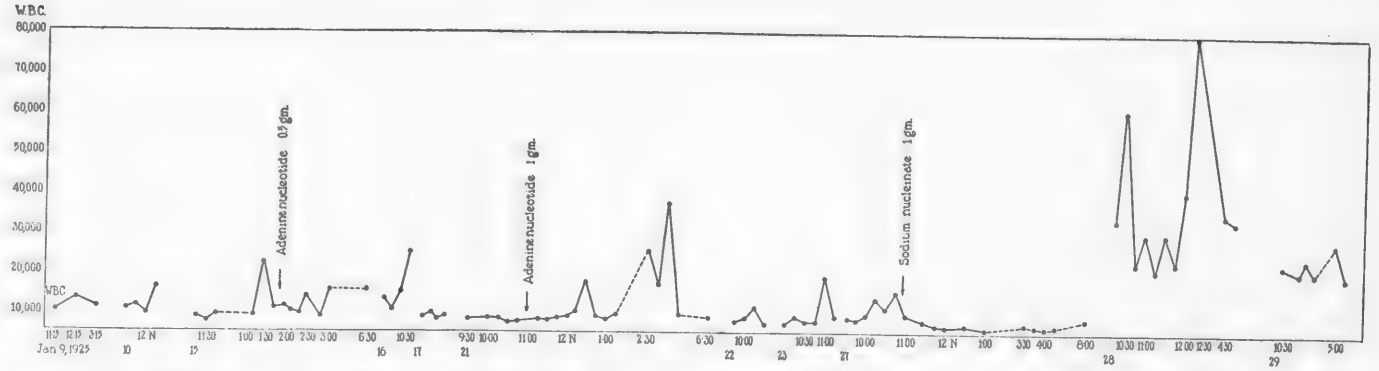


CHART 11. Rabbit 11, T 27. Adenine nucleotide (Dr. Henry Jackson, Jr.) in a dosage of 0.5 gm. was ineffective. One gm. in the same animal 6 days later produced an appreciable rise over the preliminary level within 1½ hours, and after 4 hours a count of 37,100. After another 6 days 1 gm. of sodium nucleinate induced a typical leucopenia (minimum 2900) lasting for more than 9 hours, a leucocytosis (maximum 79,500) following on the succeeding 2 days.

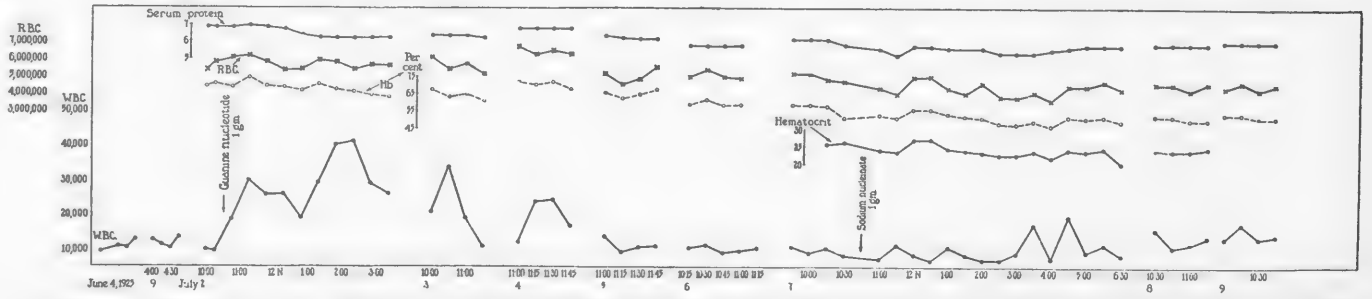


CHART 13. Rabbit 12, T 77. One gm. of guanine nucleotide produced an immediate rise in the total white count, a maximum of 41,000 being reached in 3¾ hours, an increase maintained for the 2 days following. One gm. of sodium nucleinate 5 days later gave the usual leucopenia (minimum 4700), but the leucocytosis following was only moderate in degree because of slow replacement of Myelocytes C after the preceding depletion. Both the magnitude of the initial response and the rapidity of replacement of myeloid cells show variations with individual animals. Observations on the total red cells, hemoglobin, cell-plasma ratio, and serum protein show indirectly that the blood volume did

were undertaken with adenine and guanine nucleotides, the only immediate split products of nucleic acid available at the moment.

Chart 11 records graphically the reactions to different dosages of adenine nucleotide and to sodium nucleinate in Rabbit 11. One-half gm. of adenine gave no detectable increase of cells on the 1st day and only a slightly suggestive response on the 2nd day. One gm. of the same substance 6 days later showed an appreciable rise above the control counts within 1½ hours of the injection, and a leucocytosis reaching 40,000 between 2 and 4 hours after. The same animal, when

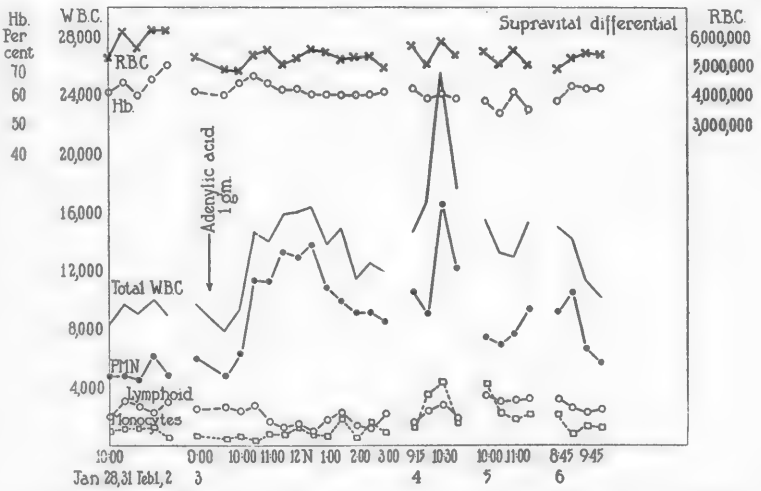


CHART 12. Rabbit 23, R 409. One gm. of adenylic acid (Dr. P. A. Levene) initiated a leucocytosis of neutrophilic origin within 1¼ hours, the curves being identical in type with those produced by the guanine nucleotide in Charts 13 and 14. Differential counts of the white cells made with the supravital technic; slight lymphopenia, no lymphocytosis; monocytic rise an individual rather than a specific response probably; red cells and hemoglobin within normal limits.

given 1 gm. of sodium nucleinate 6 days after the second dose of adenine, showed a typical leucopenia lasting for more than 9 hours with a leucocytosis the 2nd day reaching 80,000.

One gm. of adenylic acid in 15 cc. of distilled water, with just enough N/10 NaOH to put it into solution, produced a slight increase in the neutrophilic leucocytes within 45 minutes that was well marked in 1¼ hours (Rabbit 23, R 409, Chart 12). The leucocytosis was maintained during the succeeding 3 days, the highest count being recorded on the 2nd day. The differential count of the white cells made

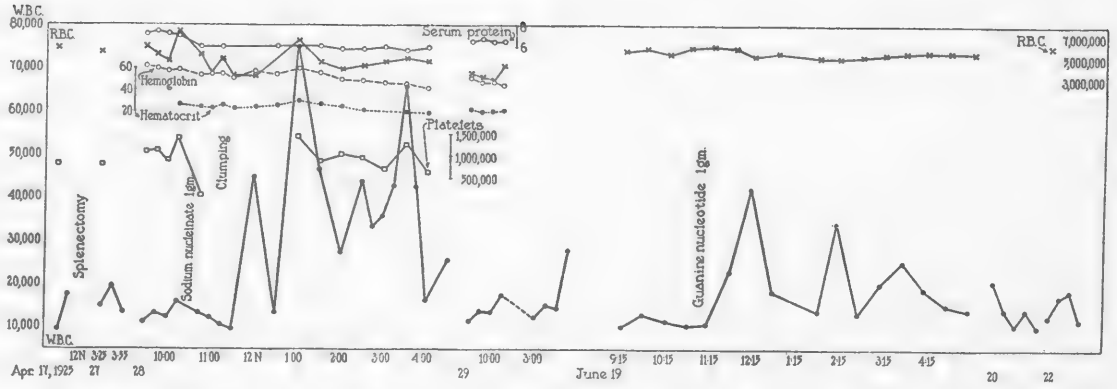


CHART 14. Rabbit 13, T 61. After splenectomy the response to sodium nucleinate and guanine nucleotide gave counts of 45,300 and 42,600 respectively within 1½ hours. The nucleinate gave the highest counts (maximum 75,200). Note the clumping of the platelets after sodium nucleinate during the time when leucopenia is usual in non-splenectomized animals.

with the supravital technic showed the specificity of the effect on the myeloid cells with the maximum a threefold increase in polymorphonuclear neutrophils. The neutrophilic leucocytes showed unusually active motility, there was a definite increase in the percentage of younger forms, and Myelocytes C and metamyelocytes were recorded in four of the counts on the day of the injection and in one count on the 2nd day. The lymphocytes showed a slight depression on the 1st day, but no lymphocytosis followed. The monocytes in this instance were increased in total number, particularly on the 2nd and 3rd days, but this would appear to be an individual idiosyncrasy of the animal instead of a part of the usual response. Red cells and hemoglobin remained within the normal limits of variation. There was no temperature reaction.

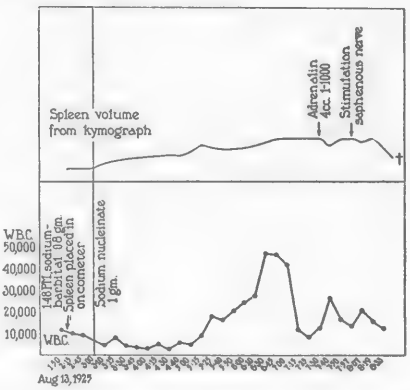


CHART 15. Rabbit 14, T 108. During the leucopenia following sodium nucleinate the spleen increases in volume. This increase is maintained through the initial period of leucocytosis, at least, and correlates with the numerical and histological evidence of greatly increased numbers of neutrophilic leucocytes in the splenic pulp. Coincident with the splenic contraction after adrenalin and after mechanical stimulation of a sensory nerve there was, in each case, a transitory increase in the white cells.

On Chart 13 may be followed the contrast in the effects of guanine nucleotide and sodium nucleinate in the same individual. The response after the administration of 1 gm. of guanine nucleotide was immediate, as evidenced by the control counts preceding. The leucocytosis reached 40,000 within 3 hours and a high count was maintained for the succeeding 2 days, the normal range being reached only on the 4th day. Five days after the guanine 1 gm. of sodium nucleinate was given, which resulted in a leucopenia lasting for more than 4 hours, the leucocytosis following being relatively low. This illustrates a latent period in the replacement of Myelocytes C after their depletion in response to the first chemotactic

substance, during which period a second chemotactic stimulus is relatively ineffective. Serum protein, red cells, hemoglobin, and cell-plasma ratio were followed in this animal, as in others, and showed no appreciable difference with the two substances. Guanylic acid in a dosage of 450 mg. (Rabbit 24, R 410) produced a proportionate immediate increase in the neutrophilic leucocytes identical with the curves shown on Charts 13 and 14 where guanine nucleotide was used. No temperature or constitutional reaction was noted.

Chart 14 shows similarity of the response to sodium nucleinate and guanine nucleotide in a splenectomized rabbit. Within 1½ hours after sodium nucleinate the count was 45,000, while within 45 minutes after guanine nucleotide the count was 23,000, and at 1¼ hours 42,000. The highest point reached under the sodium nucleinate was 75,000, 2½ hours after the injection, the highest point in this animal under guanine was 42,000, reached after 1¼ hours.

Both adenine and guanine nucleotides from two sources, in a dosage comparable to that used with sodium nucleinate, gave leucocytosis without preceding leucopenia, the effectiveness of the response being noted within the usual period of leucopenia after nucleinate. The leucopenia, described earlier, is, therefore, associated only with the more complex molecule, while the specificity of the stimulus to the neutrophilic leucocytes is retained by the less complex split products. In brief, the response of the splenectomized rabbit to sodium nucleinate is the same as that of the normal rabbit to the nucleotides.

The Effect of Sulfur and Peptone.

Since flowers of sulfur in olive oil given subcutaneously and peptone (Witte) given intravenously, have been used clinically to induce a leucocytosis, they were administered under controlled conditions to compare with the response to sodium nucleinate.

Fourteen mg. of flowers of sulfur in 2 cc. of olive oil were injected intramuscularly into Rabbit 20 (T 36). From a preliminary base line of 5500 to 7600, the counts rose to 10,000 and 11,000 1 hour and 1¼ hours, respectively, after the injection. These two counts were the only ones throughout the day going above 8700 and none were lower than 5500. This is well within the range of normal fluctuation for white cells in a rabbit in which counts are made every quarter hour during the day.

The injection of 1 gm. of Witte's peptone intravenously in Rabbit 21 (T 38) was followed by a fall in the white count from a preliminary zonal level of 9400–10,900 to 5200 in 7 minutes, coincident with a vasoconstriction; 15 minutes later, however, coincident with vasodilatation, the count was 10,400, and the count for the remainder of the day fluctuated between 6000 and 12,400.

The degree and the nature of the effects of nucleinate and nucleotides seem to be quite distinct from those observed in these last instances.

DISCUSSION.

The foregoing experiments reveal a prompt unequivocal response of the bone marrow, in the delivery of new mature neutrophilic leucocytes to the circulation, after the introduction intravenously of large doses of sodium nucleinate. However, there is a latent period in the replacement of the cells at the level of the Myelocyte C, in bone marrow, as shown through the inability to reproduce a comparable leucocytosis after too frequently repeated injections. Examination of the bone marrow confirms the rapid depletion of the myeloid foci followed by only a gradual replacement. Thus sodium nucleinate and adenine and guanine nucleotides contain stimuli capable of calling forth new neutrophilic cells so long as there is an adequate reserve of myeloid cells at the level of Myelocyte C, but they lack any factors specifically active for the maintenance of this level.

Ehrlich (51) defined chemotaxis as the attractive force, from microorganisms or toxins, for bringing cells from the large reserve always found in marrow. This term Cohnheim had acquired from the botanist Pfeiffer and carried over to the reaction involving the cells in inflammation (40). The first suggestion of a physiological factor with such potentialities was made by Horbaczewski (52) who correlated supposed digestion leucocytosis with an increase in the uric acid of the blood. However, Ewald (53) found uric acid relatively inactive in dogs in contrast to thymus and yeast nucleic acids and xanthine and guanine, the latter all inducing appreciable leucocytosis. Muir (54) assumed from his study of the marrow either a hyperplasia of myelocytes secondary to the exodus of leucocytes into the blood on the principle of overregeneration after repeated loss, as suggested by Weigert, or that the chemotactic substances directly stimulate cellular proliferation.

Sabin and Doan (33) have recently presented the reactions of bone marrow in terms of two factors, first a chemotactic factor (C) which acts directly on the myelocytes of Type C with an immediate response to a demand for increased leucocytes, such as that exemplified in the response to sodium nucleinate or guanine within 45 minutes to 2½ hours as here described; second, a maturation factor (M), not necessarily in association with the chemotactic factor, but which is

necessary for a maintained leucocytosis, through an active regeneration of myelocytes in the marrow without the latent period involved in Weigert's principle. Sabin (35), in a current review on bone marrow, discusses these factors in detail and presents a new basis upon which to approach the whole problem of the chemical control of the maturation and delivery of red and white cells from the hemopoietic centers. On such a basis as she formulates, it may be that nucleic acid and its immediate derivatives will be found to be important chemotactic factors used physiologically by the body in the maintenance of the normal equilibrium between supply and demand and even in pathological states. That a so called maturation factor is lacking in this group of substances would be the only conclusion justified from the data thus far accumulated. The myelocytic replacement here seems dependent only upon the principle of "overregeneration after repeated loss." Bacon, Novy, and Eppler (55) ascribe the leucocytic response in infection to non-specific stimuli arising from a quantitative alteration in the body metabolism rather than directly from infecting organisms, in other words, a response to the same stimuli which on a smaller scale produce the variations of normal values in the absence of infection. Under such a concept the importance of nucleic acid and of all the possible physiological stimuli is obvious.

SUMMARY.

The leucopenia induced by sodium nucleinate has been followed by repeated counts made simultaneously from the blood of a peripheral vein and from the internal organs, combined with a study of histological sections of the same organs taken with the counts. Measurements with the oncometer of changes in volume of the spleen have been correlated with the leucopenia and the leucocytosis following sodium nucleinate. It has thus been determined that the leucopenia is not the result of a vasomotor phenomenon, or of a change in blood volume, nor is it secondary to a retention of the white cells in the capillaries of lung or liver; it is due to the accumulation of neutrophilic leucocytes in the parenchyma of the spleen. That the spleen is solely responsible for the temporary depression of white cells in the general circulation under these conditions has been shown by splenectomy.

In splenectomized rabbits no leucopenia developed, but instead a leucocytosis due to a direct action on the bone marrow.

A profound change occurs in the distribution of the cells in the circulation at the moment of death. The liver, within a minute of the cessation of the circulation, shows a three- to fourfold increase in the number of white cells per c.mm., the differential count remaining unchanged. Thus estimations of the physiological distribution and redistribution of cells in the living state may be made only with the circulation unimpaired.

The injection into normal rabbits of adenine and guanine nucleotides, split products of nucleic acid, gave immediate leucocytosis of bone marrow origin similar to that observed with the more complex molecule when the spleen was eliminated.

The response of the bone marrow to chemotactic stimuli, such as those here used, may be reflected in the general circulation, through an absolute increase of young neutrophilic leucocytes, within a period of less than 1 hour. Within this brief period there takes place maturation from Myelocyte C and metamyelocyte into the early motile leucocyte, and the delivery of these just matured cells into the circulation. The response to one injection of nucleinate or nucleotide may persist into the 3rd and 4th days with a gradual depletion of the normal reserve of Myelocytes C in the bone marrow.

BIBLIOGRAPHY.

1. Sabin, F. R., Cunningham, R. S., Doan, C. A., and Kindwall, J. A., The normal rhythm of the white blood cells, *Bull. Johns Hopkins Hosp.*, 1925, xxxvii, 14.
2, *a*. Shaw, A. F. B., The diurnal tides of the leucocytes of man, *J. Path. and Bact.*, 1927, xxx, 1.
2, *b*. Fletcher, E. G., and Mitchell, A. G., Physiologic variations in the leukocytes in infants and children, *Am. J. Dis. Child.*, 1927, xxxiv, 807.
3. Stetson, R. P., Normal variations in white blood cells under conditions of minimal metabolism, *Arch. Int. Med.*, 1927, xl, 488.
4. Isaacs, R., and Danielian, A. C., Maintenance of leucocyte level and changes during irradiation, *Am. J. Med. Sc.*, 1927, clxxiv, 70.
5. Bushnell, L. D., and Bangs, E. F., A study of the variation in number of blood cells of normal rabbits, *J. Infect. Dis.*, 1926, xxxix, 291.
6. Sabin, F. R., Studies of living human blood cells, *Bull. Johns Hopkins Hosp.*, 1923, xxxiv, 277.

7. Doan, C. A., and Zerfas, L. G., The rhythmic range of the white blood cells in human pathological leucopenic and leucocytic states, with a study of thirty-two human bone marrows, *J. Exp. Med.*, 1927, xlvi, 511.
8. Mathews, A. P., Physiological chemistry, 2nd edition, New York, 1919, 161.
9. Metchnikoff, Pathologie comparée de l'inflammation, Paris, 1892.
10. Ames, D., and Huntley, A., The nature of the leucocytosis produced by nucleinic acid; a preliminary experimental study, *J. Am. Med. Assn.*, 1897, xxix, 472.
11. Milroy, T. H., and Malcolm, J., The metabolism of the nucleins, *J. Physiol.*, 1899, xxv, 105.
12. Vaughan, V. C., Novy, F. G., and McClintock, C. T., The germicidal properties of nucleins, *Med. News*, New York, 1893, lxii, 536.
13. de Paoli, E., and Calisti, A., Beobachtungen und experimentelle Untersuchungen über den Wert der Injektion des nukleinsauren Natriums bei der Prophylaxe der operativen Infektion des Peritoneums, *Mitt. Grenzgeb. Med. u. Chir.*, 1912, xxiv, 275.
14. Jacobi, W., Ueber intravenöse Injektionen hoher Dosen von Natrium nucleinicum beim Menschen, *Z. exp. Path. u. Therap.*, 1921, xxii, 266.
15. Medwin-Gardner, F. M., The precipitation of the crisis in the treatment of pneumonia, *Brit. Med. J.*, 1924, ccxlix, 748.
16. Daubenton, F., Severe case of lobar pneumonia successfully treated with sodium nucleinate, *South African Med. Rec.*, 1925, xxiii, 463.
17. Gotmakher-Redelman, A. M., New sign of Banti's disease, *Klinicheskaya Meditsma*, 1926, iv, 236 (abstract in *J. Am. Med. Assn.*, 1926, lxxxvii, 1428).
18. Antoncich, R., Bone marrow function test, *Crón. med.*, 1926, xliii, 3 and 65 (abstract in *J. Am. Med. Assn.*, 1926, lxxxvii, 1690).
19. Habetin, P., Über Funktionsprüfung des Knochenmarkes, *Wien. Arch. inn. Med.*, 1923–24, vii, 329.
20. Larsell, O., Nokes, H. T., and Phillips, B. I., Hemopoietic effect in rabbits of intravenous injection of cells, nuclei, and nucleic acids from blood of fowls, *Arch. Path. and Clin. Med.*, 1926, ii, 698.
21. Larsell, O., Jones, N. W., Nokes, H. T., and Phillips, B. I., The hemopoietic effects of intravenously injected nucleic acids, *J. Am. Med. Assn.*, 1927, lxxxix, 682.
22. Robertson, T. B., Hicks, C. S., and Marston, H. R., A comparison of the utilization of nucleic acids of animal and vegetable origin, *Wistar Inst. Bibl. Service Advance Abstr. Sheets*, 1927, No. 85, Nov. 14 (to appear in the *Australian J. Exp. Biol. and Med. Sc.*).
23. Löwit, M., Studien zur Physiologie und Pathologie des Blutes und der Lymphe, Jena, 1892.
24. Goldscheider, A., and Jacob, P., Ueber die Variationen der Leukocytose, *Z. klin. Med.*, 1894, xxv, 373.

25. Schultz, Experimentelle Untersuchungen ueber das Vorkommen und die diagnostiche Bedeutung der Leukocytose, *Deutsch. Arch. klin. Med.*, 1892, li, 234.
26. Gräff, S., Leukozytenbewegung im Blute. Verschiebungsleukozytose—myelogene Leukozytose, *Berl. klin. Woch.*, 1921, i, 84.
27. Wells, W. A., Leucocytosis and immunity, with a critical analysis of the theory of nuclein-therapy, *Med. News*, New York, 1896, lxix, 425.
28. Jones, W., Nucleic acids, London and New York, 2nd edition, 1920.
29. Roy, C. S., Physiology and pathology of the spleen, *J. Physiol.*, 1881, iii, 203.
30. Schäfer, E. A., and Moore, B., On the contractility and innervation of the spleen, *J. Physiol.*, 1896, xx, 1.
31. Doan, C. A., The recognition of a biological differentiation in the white blood cells, *J. Am. Med. Assn.*, 1926, lxxxvi, 1593.
32. Sabin, F. R., Austrian, C. R., Cunningham, R. S., and Doan, C. A., Studies on the maturation of myeloblasts into myelocytes and on amitotic cell division in the peripheral blood in subacute myeloblastic leucemia, *J. Exp. Med.*, 1924, xl, 845.
33. Sabin, F. R., and Doan, C. A., Bone marrow as an organ, *Proc. Soc. Exp. Biol. and Med.*, 1927, xxv, 121.
34. Doan, C. A., Cunningham, R. S., and Sabin, F. R., Experimental studies on the origin and maturation of avian and mammalian red blood cells, *Carnegie Institution of Washington, Pub. No. 361, Contributions to Embryology*, 1925, xvi, 163.
35. Sabin, F. R., The bone marrow, *Physiol. Rev.*, 1928, viii, (April).
36. Mueller, E. F., and Myers, C. N., Biochemical studies on the behavior of the leucocytes after intravenous administration of alkalinized salvarsan, *Proc. Soc. Exp. Biol. and Med.*, 1924–25, xxii, 95.
37. Webb, R. A., The mechanism of anaphylactic leucopenia in dogs, *J. Path. and Bact.*, 1924, xxvii, 79.
38. Bruce, D., On the disappearance of the leucocytes from the blood after injection of peptone, *Proc. Roy. Soc. London*, 1894, lv, 295.
39. Andrewes, F. W., The behavior of the leucocytes in infection and immunity, *Lancet*, 1910, ii, 8.
40. Schilling, V., Das Knochenmark als Organ. III. Funktion des Markenparenchyms und Leukozytose, *Deutsch. med. Woch.*, 1925, li, 516.
41. Oeller, *Münch. med. Woch.*, 1922, ii, 1774; 1924, i, 218.
42. Wells, C. W., Leucopenia and leucocytosis in rabbits, *J. Infect. Dis.*, 1917, xx, 219.
43. Cunningham, R. S., Sabin, F. R., and Doan, C. A., The differentiation of two distinct types of phagocytic cells in the spleen of the rabbit, *Proc. Soc. Exp. Biol. and Med.*, 1924, xxi, 326.
44. Oliver, G., and Schäfer, E. A., Physiological effects of extracts of the suprarenal capsule, *J. Physiol.*, 1895, xviii, 230.

45. Barcroft, J., Recent advances in the knowledge of the spleen, *Lancet*, 1925, i, 319.
46. de Boer, S., and Carroll, D. C., The mechanism of the splenic reaction to general CO poisoning, *J. Physiol.*, 1924, lix, 312.
47. Scheunert, A., and Krzywanek, F. W., Weiteres über die Milz als Blutkörperchen-Reservoir, *Arch. ges. Physiol.*, 1927, ccxvii, 261.
48. Viale, G., La milza organo regolatore del numero dei corpuscoli rossi e bianchi circolanti, *Arch. fisiol.*, 1927, xxv, 380.
49. Soler, F., Contribucion al conocimiento de la funciones del bazo. Sissoreis esplanica, *Rev. med. latino-am.*, 1926, ii, 126.
50. Krumbhaar, E. B., Functions of the spleen, *Physiol. Rev.*, 1926, vi, 160.
51. Ehrlich, P., Die Anaemie, in *Nothnagel, H., Spec. Path. u. Therap.*, 1898, viii, 75.
52. Horbaczewski, J., Die Ausscheidung der Harnsäure und die Zahl der Leukocyten im menschlichen Blute nach Aufnahme von Chinin, Atropin, Pilocarpin, Antipyrin und Antifebrin, *Sitzungsber. k. Akad. Wissensch., Math.-naturw. Cl.*, 1891, vol. C, pt. 3, 101, Ref. B, abstracted in *Deutsch. med. Woch.*, 1892, lxxx, 772.
53. Ewald, G., Ueber intravenöse Verabreichung von Nucleinsäure und ihren Abbauproducten beim Hund, *Z. exp. Path. u. Therap.*, 1913, xii, 348.
54. Muir, R., On the relations of the bone marrow to leucocyte production and leucocytosis, *J. Path. and Bact.*, 1901, vii, 161.
55. Bacon, D. K., Novy, F. O., and Eppler, H. H., Factors in leucocytosis, *Arch. Int. Med.*, 1922, xxx, 229.

EXPLANATION OF PLATES.

PLATE 21.

FIG. 1. Femoral bone marrow from Rabbit 6 (T 50) 1¼ hours after 1 gm. of sodium nucleinate intravenously. The sinus is surrounded with neutrophilic leucocytes in active diapedesis and to right and left of the vessel may be seen cleared areas from which the myeloid elements have been withdrawn. See Chart 6. Section 10μ. Methylene blue-eosin. × about 750.

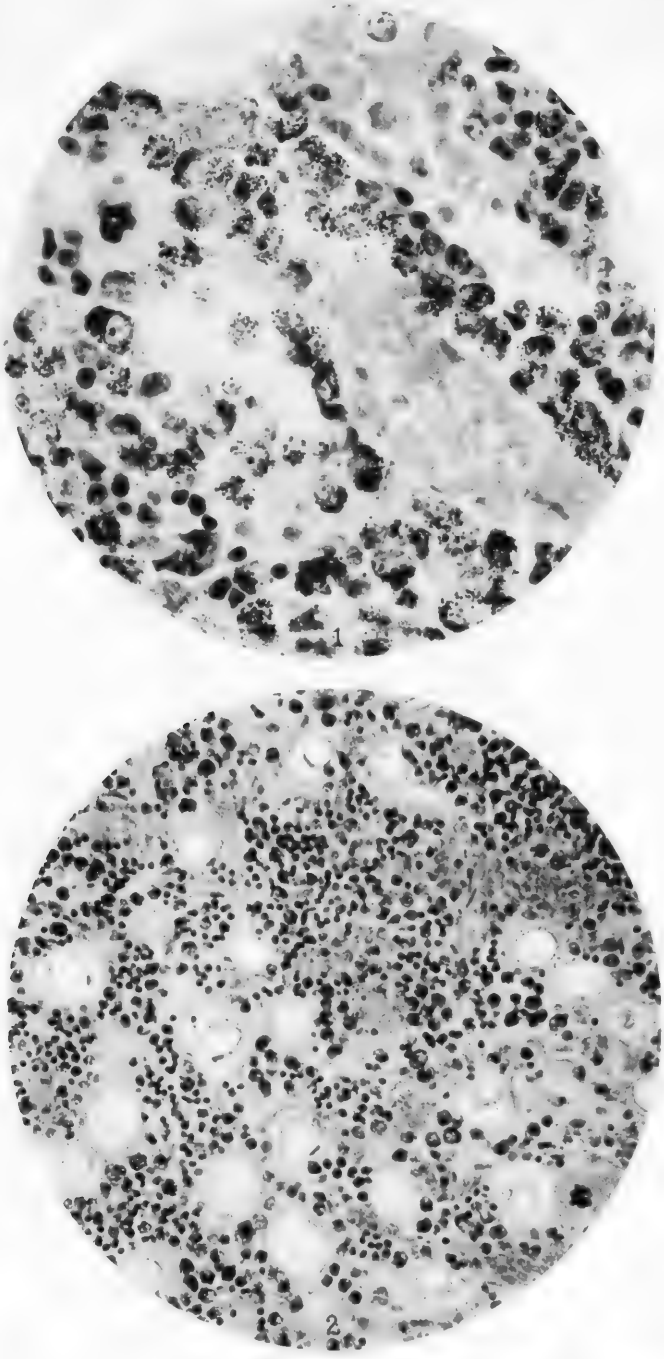
FIG. 2. Femoral bone marrow from Rabbit 22 (T 33) 18 hours after a second dose of 1 gm. of sodium nucleinate, the first having been given 4 days before. There is a decrease in size and number of fat cells in inverse proportion to the changes in the myeloid foci. It will be noted that the cells have not yet occupied all of the space made available for their development by the regression of the fat. Section 10μ. Methylene blue-eosin. × about 270.

PLATE 22.

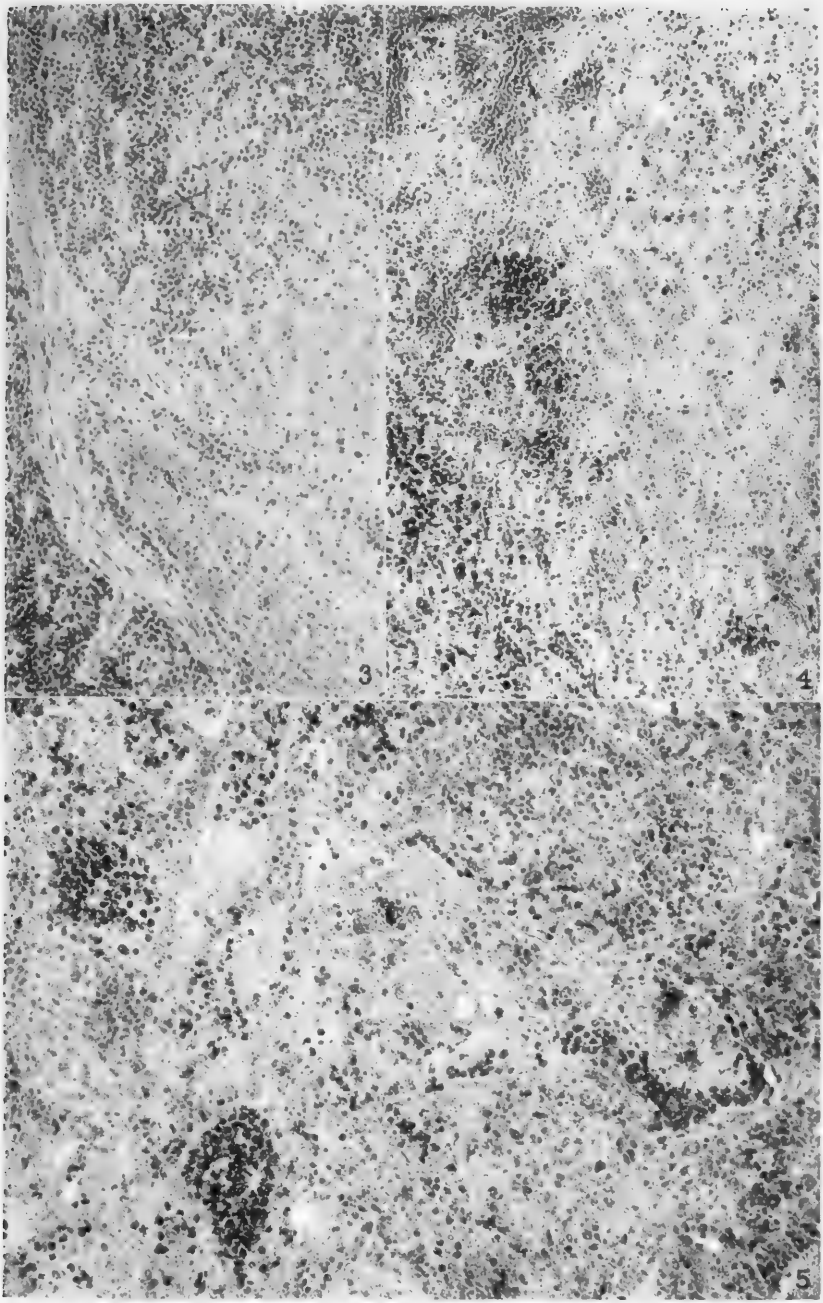
FIG. 3. Normal spleen from Rabbit 4 (T 49) 10 minutes before 1 gm. of sodium nucleinate was given intravenously. See Chart 4, Point 1. Section 10μ. Methylene blue-eosin. × 190.

FIG. 4. Sample of spleen from same experiment as Fig. 3, 1 hour after 1 gm. of sodium nucleinate intravenously. Note the beginning appearance of foci of neutrophilic leucocytes in the parenchyma. The darker cells are the neutrophilic leucocytes. The diffuse scattering of individual neutrophils throughout the pulp is not so easily recognized at this magnification. See Chart 4, Point 3. Section 10μ. Methylene blue-eosin. $\times$ about 190.

FIG. 5. Sample of spleen from Rabbit 19 (T 31) 8 hours after 1 gm. of sodium nucleinate. Leucopenia had lasted for 5 hours, followed by 3 hours of leucocytosis. The maximum accumulation of neutrophilic leucocytes in the splenic parenchyma is still maintained. The section shows definite foci of leucocytes as well as numerous neutrophils scattered throughout the pulp. The darker cells are the neutrophilic leucocytes. 10μ. Methylene blue-eosin. $\times$ about 190.



(Doan, Zerfas, Warren, and Ames: Leucopenic and leucocytic states.)



(Doan, Zerfas, Warren, and Ames: Leucopenic and leucocytic states.)

ALLERGIC IRRITABILITY.

IV. The Capacity of Guinea Pigs to Produce Antibodies as Affected by the Inheritance and as Related to Familial Resistance to Tuberculosis.

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(Received for publication, November 7, 1927.)

Wright and Lewis (1) found that certain inbred families of guinea pigs differed in their resistance to experimental tuberculosis. The work as published has been carefully repeated and considerably extended by them with particular reference to amplifying the results of cross-breeding the various families. It thus became apparent that the observed variations in familial resistance must depend on the varied inheritance of a number of factors or factor groups. The operation of at least three or four separately inherited qualities was suggested by the genetic results.* An analysis of the known physiological and structural pecularities of the families was presented by Wright and Lewis (1) and later in great detail by Wright (2, 3), it being shown that such qualities as color, ability to grow at a certain rate, weight at a certain age, fertility as measured either by the number of young born or the percentage of young raised to the weaning age, or, finally, general vigor as estimated by these several qualities combined could be of but minor significance with relation to resistance. Thus of the total observed variation in resistance between the families about 40 per cent appeared to be dependent on characteristics which can be accounted for at present. All of the above characters combined account for but 7 per cent out of the 40 per cent mentioned leaving somewhat over 30 per cent as due to inheritance. It might

* It is expected that the amplifying data here referred to will be published in detail in the near future.

therefore, within a range of the observed variation of about 30 per cent, be hoped to discover by appropriate experiment one or more inheritable qualities which could be considered influential as determinants of resistance. The present authors set themselves this problem.

Consideration made it clear that our problem was intimately involved with the uncertainties of knowledge in regard to the natural resistance to infectious diseases in general and tuberculosis in particular. It has been our plan to fix on certain qualities, which might conceivably be influential, and to determine for each so far as possible the variability of the inbreds by family. As the result of a succession of such studies it is hoped to piece together a picture which may suggest either the inherited qualities concerned or more definitive experiments to reveal them.

Among the general biological reactions considered in this connection is that of the capacity of animals to acquire an artificial immunity or sensitization in response to treatment with antigenically active materials, that is to say their *allergic irritability* (4–6). For it must be true generally with infectious diseases that the initial stages of infection elicit immunity responses and that these influence the course of the disease, its character, and termination.

In the light of the great accumulation of knowledge with regard to specificity of immunization reactions it is possible on one view to presuppose that the quantitative relations within any such group of animals as that under consideration might vary for each antigen and that the only significant reactions for our purpose would be those connected with the tubercle bacillus or its products. But at the time this problem was taken up it was quite doubtful whether the guinea pig had ever been successfully immunized against tuberculosis, and it seemed that the effort to acquire the desired information directly would be attended with many uncertainties, if it were indeed practicable in any degree. Provisional experiments in this direction were undertaken however and may be reported upon later.

On the other hand it seemed not impossible that while immunity reactions speaking generally are so highly specific, the underlying qualities in the animal upon which they depend might be much less or not at all so; that in other words within any species an animal easy to immunize with one substance might be relatively easy to immunize

with any other, or with some others. As to this the literature afforded little information and hence experiments were undertaken with various immunity reactions which were easy to execute. We have thus studied the formation of hemolytic amboceptor for sheep and beef corpuscles, agglutinins for *Bacillus typhosus* and *Bacillus abortus* (Bang), and the reaction of anaphylaxis to sheep red blood corpuscles and to horse serum. From the beginning of the work the results have been suggestive with relation to our problem. Incidental results of considerable interest with reference to allergic irritability in general were also obtained and have been the subject of publications by Lewis and Loomis (4–6).

It was found (4, 6) that preexisting infectious disease, especially tuberculosis had a very marked stimulating influence on the quantity of antibodies produced by antigens unrelated to those involved in the disease process. This observation is pertinent to the present discussion in that it implies that a more or less common mechanism underlies the highly specific reactions of immunity with which we are more familar and on which we are technically dependent for information in this field.

We found (5) that when the complete experiment used for the classical demonstration of the phenomena of anaphylaxis was carried through on our guinea pigs the outcome varied by families and that there was a parallelism between the reactions of the families in this experiment and their resistance to tuberculosis as determined by Wright and Lewis. Those most resistant to the disease were the least responsive in the anaphylaxis experiment.

The series of papers (particularly Lewis and Loomis (4)) also give in detail the methods which we have employed in the estimation of antibody production, some discussion of the principles underlying our problem, and a review of the pertinent literature.

Our present purpose is to present and discuss briefly such observations as we have accumulated bearing on the varying capacities of the inbred families of guinea pigs to produce antibodies, keeping in mind especially the question as to whether there is any parallel to be observed between this capacity and resistance to tuberculosis.

EXPERIMENTAL.

In the first instance groups of animals from each family were injected simultaneously with sheep red blood corpuscles and a heat-killed culture of *Bacillus typhosus*. The antibodies, anti-sheep amboceptor and anti-*typhosus* agglutinin.

TABLE I.

Order of Families Relating Antibody Production to Resistance against Tuberculosis.

T. B. resistance	Anti-sheep hemolysin		Anti-beef hemolysin		*B. typhosus* agglutinin		*B. abortus* agglutinin		*B. abortus* fixation
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	
35	35 (616)	35 (3000)	35 (524)	2 (487)	13 (400)	13 (60)	35 (32)	35 (94)	35 (940)
2	13 (280)	13 (2700)	2 (343)	35 (460)	35 (93)	35 (13)	2 (17)	2 (80)	2 (658)
32	2 (182)	2 (1200)	13 (331)	13 (280)	2 (90)	2 (13)	13 (15)	13 (61)	13 (237)
13	32 (92)		32 (226)	32 (220)	32 (20)	32 (6)	32 (14)	32 (14)	32 (110)

TABLE II.

Family No.	Individual No.	Natural amboceptor undiluted serum	Acquired amboceptor—dilutions					
			1:3	1:5	1:10	1:20	1:30	1:40
35	1	++++*	0	0	0	0	0	0
			++++	++++	++++	+++	+	0
(1/100)	2	++	++++	++++	++++	+++	+	0
			++++	++++	++++	+++	0	0
	3	±	++++	++++	++++	+++	+	
			++++	++++	++++	+++	++++	++
	4	++++	0	0	0	0	0	0
			++++	++	0	0	0	0
(1/1000)	5	±	0	0	0	0	0	0
			d.	–	–	–	–	–
	6	0	0	0	0	0	0	0
			++++	++	0	0	0	0
13	1	0	++++	++++	++++	++++	++	0
			++++	++++	++++	++++	++	0
(1/100)	2	++++	++++	++++	++++	++++	+++	++
			++++	++++	++++	+++	++	0
	3	++++	0	0	0	0	0	0
			++	0	0	0	0	0
	4	0	0	0	0	0	0	0
			0	0	0	0	0	0
(1/1000)	5	0	0	0	0	0	0	0
			+	0	0	0	0	0
	6	0	0	0	0	0	0	0
			+	0	0	0	0	0
32	1	±	0	0	0	0	0	0
			d.	–	–	–	–	–
(1/100)	2	+++	++++	++++	+++	+	0	0
			d.	–	–	–	–	–
	3	±	0	0	0	0	0	0
			0	0	0	0	0	0
	4	±	+	0	0	0	0	0
			d.	–	–	–	–	–
			+	0	0	0	0	0
(1/1000)	5	++++	0	0	0	0	0	0
			+	0	0	0	0	0
	6	0	0	0	0	0	0	0

*++++ = complete hemolysis; 0 = no hemolysis; d. = dead.

Of the two readings shown for each individual the first is for the 9th, the second for the 22nd day after injection.

TABLE II—*Concluded.*

Family No.	Individual No.	Natural amboceptor undiluted serum	Acquired amboceptor—dilutions					
			1:3	1:5	1:10	1:20	1:30	1:40
2	1	0	0	0	0	0	0	0
			++++	++	0	0	0	0
(1/100)	2	0	0	0	0	0	0	0
			+++	+	0	0	0	0
	3	0	0	0	0	0	0	0
			+	0	0	0	0	0
	4	++++	0	0	0	0	0	0
			0	0	0	0	0	0
(1/1000)	5	0	+	0	0	0	0	0
			0	0	0	0	0	0
	6	+	0	0	0	0	0	0
			+	0	0	0	0	0

were determined on the 9th day after the first treatment. The same animals were then treated simultaneously with beef red blood corpuscles and a heat-killed culture of *Bacillus abortus* (Bang). Again the respective amboceptor and agglutinin titres were determined on the 9th day after injection. There were three animals in each family group. Table I in the 1st column under each subhead shows the results obtained in this experment. The experiment was repeated using groups of ten animals for each family. The results are shown in the 2nd column under each subhead. The arrangement is by family numbers in order of decreasing titre from top to bottom. The actual average titres are shown in parenthesis and where these are considered to differ by insignificant amounts they are bracketed together. In connection with the second experiment the titres of the sera for *Bacillus abortus* in the complement fixation reaction are also shown as they emphasize the significance of the differences among sera of low agglutinin titre (last column Table I).

This type of experiment was carried through once more using beef corpuscles and the heat-killed culture of *Bacillus bovisepticus* (Strain Pn. I). Agglutinins did not develop and Family 32 was not represented. The result with reference to anti-beef hemolytic amboceptor in the terms used in the table was first, 35 (3000); second, 13 (2700); third, 2 (1200).

Several experiments were done in which much larger and repeated doses of sheep cells were given. It was thought that the higher titres of amboceptor so induced might give better definition to the place of the families. This proved not to be the case. In general either Family 13 or Family 35 were the high producers but the irregularities rather than the resemblances were emphasized in this type of ex-

periment. Family 32 was represented in but two of this group of experiments and it took a high place being highest once and second to Family 35 once.

Since the exaggeration of the antibody production seemed, as has been said, to emphasize irregularities in the relations and probably to introduce additional factors it was thought possible that the repetition of the study using the minimal amount of antigen necessary to a response might be instructive. Two experiments were conducted on this basis as follows:

In the first of these, six animals were taken from each family. They were tested individually for natural hemolysin and then injected with washed red corpuscles of the sheep in amount equivalent to 1/100 cc. and to 1/1000 cc. of whole defibrinated blood, three animals of each family being used for each dose. The animals were bled on the 9th day and on the 22nd day after injection. The titres of the serum for anti-sheep amboceptor were determined in the usual way in the presence of an excess of guinea pig complement. The results are shown in Table II.

Findings.

It was found that with reference to our problem no significance is to be attached to the presence of natural amboceptor. This is very small in amount at most and frequently undemonstrable. Each of the families shows instances of its presence in maximum and its complete absence. When present it apparently does not influence the formation of specific amboceptor as instances appear of animals with the maximum of natural amboceptor giving no increase on treatment and of those with no natural amboceptor giving a good response to the injection.

Considering the acquired antibodies by families two clearly defined groups appear. The animals of Families 35 and 13 produced much more antibody than did those belonging to Families 2 and 32. Within the groups Family 35 did somewhat better than did Family 13. No distinction of value appears between Families 32 and 2.

This experiment was repeated in its essentials with the same general result. The grouping by families appeared as before. The only difference was that within the groups on repetition Family 13 produced definitely more amboceptor than Family 35.

The animals used in the second experiment were now given a second injection of 1/100 cc. of sheep corpuscles. The results were in accord with those previously obtained when repeated doses or larger amounts of corpuscles were administered. In this response to a second injec-

tion Family 13 continued to lead but the level of Family 35 was essentially equaled and actually overtopped by a small margin by Families 2 and 32.

In connection with some of the experiments reported in Table I and those when larger and repeated injections of antigen were given attention was paid to the curve of antibody production. As already reported (4) it was found that in the guinea pig the curve of anti-sheep amboceptor production in response to any injection usually presents two peaks, one at about the 9th the other at about the 22nd day. Attention has been directed to possible variants in this curve with relation to family. In the main features and especially with reference to first injections no suggestion of any difference has appeared. The maxima are found at the 9th and 22nd days for each family. There is something to suggest that with reference to the second or subsequent injections the rise to and fall from the maxima is somewhat more abrupt in the case of Family 32 as compared to the others.

Interpretation.

Classified by their capacity to produce hemolytic antibodies for sheep or beef corpuscles, or agglutinins for *Bacillus typhosus* and *Bacillus abortus* (Bang) the inbred guinea pigs we have been able to study fall into two clearly defined groups; Families 13 and 35 are high producers, low are Families 2 and 32. It is found that this grouping appears most clearly when minimal doses of antigen are used in the form of a single treatment (Table II). When the amount of antigen is somewhat increased the grouping is still presented, possibly somewhat less clearly (Table I). When the treatment is in the form of much larger single doses, or repeated injections even of those minimal in amount the groups are no longer well defined. It seems justifiable to consider that the responses to the first minimal impact with antigen most truly represent the latent capacity of the animal to react with antibody production.

In the case of injections repeated at intervals it must be true that the result is a complex made up of the initial response on the basis of latent capacity and a secondary response on the basis of a stimulated

or acquired capacity. There is no *a priori* reason why this secondary reaction should not develop on the basis of inherited characteristics as the primary one seems to do. With equal probability in anticipation it might be foreseen that the reactions of the two periods should either be closely correlated (that is dependent on the same set of characters) or quite independent.

In general if the correlation were close the results in the two periods should be of the same order and it might even be expected that those of the second period with high absolute titres of antibody should be the more distinctive. That this is not the case lends something to the view that the reaction characters underlying the two phases are at least partially distinct.

The regularity with which Family 32 takes a low place when given a single treatment of antigen in small and moderate amounts suggests that some special significance should be attached to those instances when in response to repeated treatments this family takes a high place. It may well be that the reactions peculiar to the second phase of antibody productions are also conditioned by inherited characters but somewhat different from those influential in the initial response.

It seems very likely however that the results in the second period are rendered obscure and irregular by some other influence. A scrutiny of the individual records suggests that individual differences in reaction may be relatively more pronounced in this phase. The number of animals for which individual records are available is too small to enable us to decide this point. Many of the data included in the study were based on results with serum pooled by families rather than on the average of individual tests.

The order of familial resistance to infection with the tubercle bacillus, as determined by Wright and Lewis (1), from high to low is 35, 2, 32, 13. Within this group 2 is more like 35 and 32 more closely allied to 13. Comparing the order of tuberculosis resistance with the allergic irritability, arranging the families from high to low in each case and indicating the grouping by parenthesis, gives the following:

Tuberculosis resistance (35, 2) (32, 13).
Allergic irritability (35, 13) (2, 32).

It is therefore apparent that allergic irritability as defined or recognized by the capacity to produce antibodies against the antigens here used is not perfectly correlated with and hence cannot be wholly responsible for the differences in resistance. With so much understood there are still observable relations which seem to be of interest. Thus if we were to set aside Family 13 in both cases the order of the remaining families would approximate 35, 2, 32 in each relation. This seems to be sufficient to suggest that the latent capacity to produce antibodies freely may be of some significance to the natural resistance against tuberculosis. Family 13 would in this interpretation be regarded as peculiar. In later papers we hope to be able to show that this family is also peculiar in other respects which may be significant.

In our previous paper in which anaphylaxis was used as a criterion for the allergic irritability the agreement between the results and the order of resistance was also suggestively close. If it be considered that the reaction of anaphylaxis is solely determined by the abundance of antibodies produced in response to the sensitizing injection there is developed a definite conflict between results of the two types of experiment. For whereas Family 13 which is now shown to be generally a high producer of antibodies was also found the most susceptible in the anaphylactic reaction, Family 35 which is also a high producer of antibodies was definitely the most resistant to anaphylactic shock. Two equally reasonable explanations suggest themselves. Either the two kinds of antibodies are independently produced, or there is in the anaphylactic response some other and hitherto unrecognized factor which serves to protect Family 35 in spite of its presumably high content of antibodies. This matter could be decided were it possible to make satisfactory quantitative determination of the passive transfer of anaphylaxis. This we have not so far been able to do and the nature of the differences revealed by the experiments in question remains problematical.

It may not be redundant to point out once more that in this work whenever the inbred families are found to differ significantly with respect to any quality or capacity this is to be regarded as evidence *per se* that the character is influenced by the inheritance either directly, or indirectly through other inheritable interacting characters. As

all inheritance is now held by most specialists to be Mendelian such qualities and capacities are likewise to be thought of as subject to Mendelian principles. The direct determination of the Mendelian attributes however must depend on the results obtained with intercrosses between the families. In the instance here considered, the ability to produce antibodies, we have so far been unable to trace significant differences through the reactions of the crossbreds. So that while we are able to conclude that the allergic irritability as expressed in the capacity to produce antibodies is high or low according to the inheritance we can only infer that the inheritance itself is in accord with the Mendelian conception.

SUMMARY.

The allergic irritability of closely inbred guinea pigs as represented by their capacity to produce hemolytic antibodies for beef and sheep corpuscles, and agglutinins for *Bacillus typhosus* and *Bacillus abortus* (Bang) differs by families and therefore is at least partly dependent on inherited characteristics.

These differences show an imperfect but suggestive correlation with the differences in resistance of the same families to inoculation tuberculosis as previously determined by Wright and Lewis.

The differences in antibody production also show an imperfect correlation with the differences in response in the anaphylactic reaction complex as previously determined by Lewis and Loomis.

These studies suggest very strongly that the allergic irritability is one of the several inheritable characters which form a partial basis for the natural resistance to tuberculosis.

The antibody-producing capacity is only satisfactorily defined when minimal or moderate amounts of antigen are used and this in single treatments. The irregularities in experimental result when repeated treatments or very large single treatments are used suggest that antibody production in the second or "acquired capacity" phase may rest on a somewhat different fundamental basis than the latent or potential natural capacity. There is some very slight evidence that production in the second phase may also be influenced by inherited qualities.

BIBLIOGRAPHY.

1. Wright, S., and Lewis, P. A., *Am. Naturalist*, 1921, lv, 20.
2. Wright, S., *U. S. Dept. Agric., Bureau Animal Ind., Bull. 1090*, 1922.
3. Wright, S., *U. S. Dept. Agric., Bureau Animal Ind., Bull. 1121*, 1922.
4. Lewis, P. A., and Loomis, D., *J. Exp. Med.*, 1924, xl, 503.
5. Lewis, P. A., and Loomis, D., *J. Exp. Med.*, 1925, xli, 327.
6. Lewis, P. A., and Loomis, D., *J. Exp. Mèd.*, 1926, xliii, 263.

ULCERATIVE TYPES AS DETERMINED BY INHERITANCE AND AS RELATED TO NATURAL RESISTANCE AGAINST TUBERCULOSIS: AN EXPERIMENTAL STUDY ON INBRED GUINEA PIGS.

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Plate 23.

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Wright and Lewis (1) found that inbred guinea pigs differed by families in their natural resistance to inoculation tuberculosis. It seemed probable to the present authors that the animal material was suitable for the determination of some at least of the physiological attributes underlying natural resistance to tuberculosis. We have previously reported studies in this general direction on the allergic irritability of these animals and of tuberculous animals in general (2–5). The present report concerns the reaction of the tissues, more particularly, but not exclusively, the skin.

The experiments are unique so far as we know both in the method of observation and the character of the animal material and there is no literature bearing directly on the work. On the other hand, the results revivify in our minds discussions of diverse questions in physiology and pathology: constitution, diathesis, predisposition and local or tissue resistance, which have been more or less continuously under observation and discussion by medical men and students of inheritance for generations. It seems proper therefore to report the experiments as directly and completely as possible and to defer reference to the observations and speculations in these various fields to later pages where the bearings of the work can be analyzed.

OBSERVATIONAL PART.

The animal material was that previously used by Wright and Lewis (1) consisting of five families of guinea pig which had been inbred by continuous brother and sister mating for a long succession of generations. The families are designated by number as 35, 2, 32, 13 and 39. They decrease in resistance to tuberculosis in the order stated.

It was thought necessary, in the course of the general experiments designed to determine resistance as measured by length of life after inoculation, to vary the method of inoculation in order to make certain that the results were independent of technical details. In due course, therefore, series of animals were inoculated directly into the skin with such amounts of virulent tubercle bacilli that death from tuberculosis might be expected in about 3 months.

The constant result of the intracutaneous inoculation is the formation of a papule which increases to a nodule of considerable size. Sooner or later the center of this nodule undergoes an ulcerative process of greater or less extent. From the time the lesion is fully developed it displays processes of healing. The ulcer shrinks and may heal over. If most of the nodule has become involved in the ulcer the whole lesion may heal and leave behind a hairless linear scar. The more usual course perhaps is that the ulcer and nodule, both reduced in size, become quiescent or stagnant and persist through the life of the animal.

When this reaction was understood it was thought that there might be some significant correlation between the development of the local lesion and the length of life of the *individual* animal. And indeed there is a relation which might be measurable between length of life and the healing process above referred to but it seems of little present significance, being apparently based on the fact that the healing process is a slow one and that only those animals which live a long time give it a chance to develop fully. The tendency to heal is exhibited by nearly if not quite all the lesions about the 3rd week. It is apparently consecutive to the discharge of matter from the nodule through the ulcer.

We have studied with care the early periods in the development of the cutaneous lesion to see whether there are discernible characteristics which might, by tendency at least, render predictable the length of life of the *individual*. Such have not been found.

When the lesions are examined with reference to the *family* there are easily recognizable qualitative differences and it has been possible to express these roughly in figures so that they acquire a certain quantitative significance. The characteristics are best developed in the case of Families 35, 13 and 39, which it will be recalled are at the extremes in resistance, 35 being the most resistant, 13 being low and 39 the lowest. The work with Families 35 and 13 as concerns this matter has been exhaustive, and warrants quite final opinions. Family 39 was available in inadequate numbers early in the observation period and more recently has been unavailable. The characteristics presented by it in certain instances were definite and easy to discriminate, but gain most of their weight in formulating conclusions from the fact that they have never appeared among the very large numbers studied from other families.

When a number of animals of the two families, 35 and 13 (we have usually used twenty of each family for an experiment), are inoculated intracutaneously at the same time and with all possible care to make the inoculation uniform as to amount and location, and when these are subjected to a daily examination, differences are definite by the 10th day and continuously so thereafter.

First recognizable is the fact that the papules and nodules are somewhat larger in the case of Family 13. The ulcerative stage also appears somewhat earlier in this family. When the lesions have reached full development at about the 20th day these general differences in size persist, and do so for as long as any considerable number of the animals remain alive with unhealed lesions.

Examined at the period of maximum size the lesions are quite different in other respects. The lesion in the case of Family 35 is firm and very sharply circumscribed on palpation. The ulcer, usually centrally situated in the lesion, is surrounded by a wide margin of firm tissue. The ulcer itself is dry, relatively shallow, is saucer shaped when round, with sharply defined margins, the whole ulcerated surface being clearly visible.

In the case of Family 13 it is frequently difficult to define the lesion by palpation. The ulcer occupies a much larger proportion of the whole lesion. The rather narrow but thick and raised margin of the ulcer is soft and edematous. The base of the ulcer is apt to be moist,

and the ulcer seems deeper because of the raised or thicker margin. The margin is excoriated and inspection does not reveal the full extent of the ulcerated area because of undermining at the edge.

The illustrations (Fig. 1, Family 35, and Fig. 2, Family 13) show these characteristics fairly in so far as they are determinable on inspection. In the plates the lesion in the case of Family 13 *appears* to be more definitely circumscribed and no larger than that pertaining to Family 35 owing to the fact that palpable qualities could not properly be considered by the artist.

It would seem possible to apply the terminology of the surgeon to these lesions and to describe them both as active, healthy ulcerations, Family 35 presenting a restrained type, Family 13 an unrestrained and tumid one. It would probably be possible in such an experiment at the period of full development to identify the family in about 75 per cent of the cases by an inspection of the lesions. The failures in identification would be almost if not quite altogether cases where Family 13 would be wrongly assigned to Family 35 on this basis.

When in one such group experiment, six animals of Family 39 were included a condition developed which is well represented in Fig. 3. During the first 20 days there was nothing to distinguish these animals from those of Family 35. Thereafter their lesions developed and maintained till death, in increasing degree, a scaly or scabby margin to the very dry ulcer. When the scabs were removed patches of excoriation were found under them and the lesion continued to spread, not as the extension of a solid nodule with a widening central ulceration, but as minute ramifying excoriating ulcerations in the more superficial layers of the skin, covered by the scaly scabs described and pictured.

In another experiment comprising twelve animals of this family but four of them developed similar lesions and in these the peculiarities were less pronounced. Animals have not been available for further tests. The significance of the result seems beyond question however for nothing approaching this lesion in type has been seen either in any of the animals of the other families or in any stock animal intracutaneously inoculated. In surgical terms this lesion could be described as an indolent excoriating ulceration.

Family 13 has presented another distinctive departure from the

other families which is of significance in the present connection. The inoculations have been practiced in or near the midline somewhat below the umbilicus. The inguinal lymph nodes on both sides are secondarily involved without exception and are usually symmetrically enlarged. In the case of this family (13) and in no other it has happened not infrequently that the inguinal lymph nodes when quite large have become adherent to the skin and this has been followed by ulceration and the later formation of a persistent discharging sinus. Moreover in many instances in this family, but in no other, smaller secondary nodules have formed along radiating lines (presumably lymphatic) between the site of the initial lesion and the inguinal nodes. These have reached a diameter of several mm. and then broken, leaving ulcers showing a considerable persistence but with a general tendency to heal.

With these types clearly in mind we have repeatedly surveyed Families 2 and 32, as well as various crossbreds having their parentage in the five family lines in all possible combinations. We have been unable to recognize any other type of lesion and *on a purely qualitative basis* we have been unable to definitely place any other family or group with reference to these types. In other words, if we had had only Families 32 and 2 and the various crossbreds, while we could have made out the types of lesion characteristic for Families 35 and 13 they would have appeared as the extremes in a variable series of individual reactions and we should not have been able to assign type to group with reference to inheritance or resistance to tuberculosis. As will be pointed out in succeeding paragraphs rough qualitative considerations suggest a definite evaluation of Families 2 and 32 also, but with the crossbreds we have been able to make no group discrimination.

In order to arrive at a roughly quantitative description of the differences we have measured the lesion in its longest direction and in the direction of a line at right angles to the longest, both in mm., and have then multiplied these together to give a value in square measure which we have termed the area. Since all the contours are rounded and the lesion is often quite round this is not a true area but an arbitrary value. The true area would be very difficult to determine in view of the irregularities in contour. The arbitrary area agrees well with the qualitative characteristics. To those accustomed to evaluate tumor forma-

tion it may appear that thickness should also be estimated and the volume be the basis for comparison. The lesion however differs from a tumor in that at the time the ulcer is established there is a discharge of matter and thereafter volume even if determinable would, we think, be less representative.

The arbitrary area of the ulcer is also determined in a similar way, and at each observation period the percentage of animals showing ulceration, and later in the experiment, complete scar formation is also noted.

The data of one such experiment in which four families were used are collected in Table I.

If the figures as given in the table for Families 13 and 35 are contrasted it is seen that for each observation period the nodules and ulcers are larger in the case of 13 than that of 35. It is also apparent that the ulcer in 13 is larger in proportion to the total area of the nodule. The percentage of animals showing ulcers is always greater for this family except during the midperiod when 100 per cent show ulcers in both. From the 68th day onward when complete healing is in evidence the number of animals showing residual scars is always much less in 13 than in 35. For these two families this is the third in a series of experiments similar in every controllable respect and with the same outcome. Family 13 then seems always to give the more severe local lesion in response to equal injections of tubercle bacilli into the skin.

With respect to Families 2 and 32 the table reveals difficulty in assigning them a definite place. Up to the 40th day they are very much alike and if the size of the ulcer alone could be considered they would in this period be clearly intermediate between 13 and 35. For the three observation periods, 19, 26 and 34 days the total lesion is even smaller than in the case of 35. The percentage of ulcers is larger than for 35. From the 40th day onward these families diverge, No. 2 rather definitely taking its place with No. 13 and No. 32 with No. 35.

The whole result as to these families would seem to place them as intermediate in respect to the character of their local lesions between Families 35 and 13, with Family 32 showing a more definite resemblance to Family 35 and Family 2 a greater likeness to Family 13.

It will be noted for all families that in the later periods of observa-

TABLE I.

Days	Lesion	Reactions by families			
		No. 35	No. 13	No. 2	No. 32
12	N	38	46	42	45
	U	3	6	4	5
	Per cent U	32	56	45	61
19	N	62	68	56	55
	U	8	13	10	10
	Per cent U	87	100	95	100
26	N	48	55	45	45
	U	6	12	9	8
	Per cent U	100	100	100	100
34	N	47	54	51	42
	U	4	11	10	9
	Per cent U	100	100	100	100
40	N	46	60	57	51
	U	3	13	11	10
	Per cent U	88	100	100	100
47	N	38	66	62	44
	U	3	14	11	8
	Per cent U	81	100	100	94
54	N	38	69	66	43
	U	3	14	11	6
	Per cent U	54	100	98	91

In the first vertical column (days) the elapsed time after inoculation is given in days. In the second vertical column there are indicated for each observation period, 1st, the average size of the nodule in sq. mm. (N); 2nd, the average size of the ulcer in sq. mm. (U); 3rd, the percentage of those animals remaining alive at any observation period which show ulceration (per cent U); and 4th, for the observation periods 68 days and succeeding, the percentage of those animals remaining alive showing only residual scars (per cent S O). Comparison of the family groups for each character observed and for each observation period is obtained by reading left to right the remaining columns headed with the family numbers 35, 13, 2, 32.

TABLE I.—*Concluded.*

Days	Lesion	Reactions by families			
		No. 35	No. 13	No. 2	No. 32
61	N	18	54	57	18
	U	3	12	13	5
	Per cent U	54	95	100	78
68	N	17	43	54	12
	U	5	9	10	4
	Per cent U	21	73	90	50
	Per cent S O	25	2	2	18
75	N	19	30	52	10
	U	4	7	11	2
	Per cent U	19	53	88	28
	Per cent S O	53	7	5	19
87	N	17	40	61	18
	U	2	6	12	4
	Per cent U	28	61	88	57
	Per cent S O	33	2	5	12
96	N	21	30	58	10
	U	4	6	12	3
	Per cent U	23	44	84	26
	Per cent S O	42	2	7	32

tion there are certain irregularities in progress from week to week in the percentages showing ulcers and scars. This is due partly to deaths, the figures being calculated on the basis of the total living at any date, and partly to the fact that progress toward healing is not steady. Ulcers heal over and break down again and again before scar formation is complete. These changes affect the average size of the ulcers but little because in this late period of successive relapses they are always very small.

It was thought possible that the different characteristics of the lesions as above described might be a reflection of an immunity developing at a different rate with advancing disease. As this if true might be associated with differences in the character of the tuberculin reaction, tuberculin skin tests were made on a selected series of these ani-

mals. A few tests were done on Family 39. Families 2 and 32 were not tested. Tests of Families 35 and 13 were adequate for conclusions.

Family 39 failed to react to tuberculin in the tests made. It seems possible that this is related to the fact that these animals are so low in resistance that the period when they could become sensitive after the establishment of the disease overlaps that period late in the disease when animals as a rule fail to react. Or it may possibly have some deeper significance.

The tests on Families 35 and 13 were interesting in that the size of the local reactive lesion in the skin differed by families in about the same degree as did the size of the initial lesion due to the living bacillus, Family 13 always presenting the larger lesion. The tests do not cover all the possible relationships as the absolute sensitivity of the animals was not measured, only the character of reaction to equivalent full doses.

The result was such as to raise a question as to whether the differences in size and quality of the skin lesions were related in any specific way to the disease-producing qualities of the tubercle bacillus. As a test in control of this point the animals were injected into the skin with various amounts of turpentine and of dilutions of tincture of cantharides, agents well known to produce acute aseptic inflammatory processes. As a reaction against these materials local nodules followed by ulcerative processes were induced, and for these also the lesions were larger and more tumid in Family 13 than in Family 35.

These processes have in common the fact that as inflammations they are characterized by a pronounced focal accumulation of leucocytes particularly the polymorphonuclear types. They were controlled by comparing mild burns of the shaved skin produced by water of various temperatures applied in flat bottom vials. The results were inconstant. No differences were made out between Families 13 and 35. The lesion here is a flat shriveling crusting disc in which the ulcerative and healing processes go on under the dead epithelium and crust without the accumulation of gross nodules.

It may therefore be concluded that the differences above described between the lesions in Families 13 and 35 are not specifically related to the injury done by the tubercle bacillus, but only incidentally to this as a part of a more general reactivity. The families differ in their

reaction to inflammatory irritants, particularly perhaps to those which cause pus formation locally, whether these act very acutely (turpentine and cantharides) or more chronically (the tubercle bacillus).

The inbred families differ from each other in another respect, of interest in the present connection, which has not so far been stated either by Wright, Wright and Lewis or the present authors. When in the course of these studies we had occasion to test the effects of the intravenous inoculation with the tubercle bacillus this was done by dissecting free the jugular vein. Families 35 and 13 were used for the comparison, twenty animals belonging to each being thus operated on in this minor way at one sitting.

In order that the experiment as a whole might have a uniform beginning we had previously practiced the technique on other animals and had standardized a simple operative procedure. It was necessary to extend the animal on a suitable board, administer an anesthetic, shave the neck, make a small incision, free and lift the vein by blunt dissection, make the injection, remove the forceps and close the wound with a single stitch. The animals weighed about 300 gm., that is they were young and the tissues were relatively soft. In case the vein or any branch of it was torn it was necessary to abandon the attempt and operate on the other side. It was found that the procedure including the change of animals on the board took 5 or 6 minutes, going on at the rate of 10 or 12 an hour. The forty animals were injected after 11 a.m. of one day. They were operated on in alternate family groups of three or four animals, and in order to finish during the working day, allowing for interruption and rest periods, it was necessary to make a conscious effort to maintain a rate of work approaching the best possible. The conditions for the following observations were therefore such that incidents which under other circumstances might have been unimpressive because of their casual and occasional character became striking by reason of the enforced attention, and were checked both by alternative repetition and the time factor involved.

It was clearly observable at once that this simple operation was easier to complete in the case of Family 35 than Family 13. In 35 the tissues were stronger, there was less loose free fat in the areolar tissue both generally in the subcutis and about the veins. The vein was thus easier to find and to expose. The vein wall was definitely stouter, less easily punctured by chance and the small branches less subject to tear. The instances in which it was necessary to go to the opposite side to complete the injection were fewer.

These differences were interpreted as evidence of some constitutional divergence in the families. The animals were more closely

scrutinized from this point of view and it was evident that making due allowance for age Family 35 is built on more compact lines and is more excitable and active. This family also has a higher muscular tonus, evident on handling. The differences in nervous irritability were, it was found, well known to the attendants. Subsequent experience has shown that these differences may be obscured by conditions. They are most apparent when the diet is somewhat restricted pointing to a general difference in "feeding quality" to use the terms of the livestock man. The differences are of the character that might generally be described as those between the "lean and hungry" type and that with the obese tendency.

These qualities as between the two families considered (35, 13) may possibly be related to the observations by Wright, that among the five families 13 was marked by the best capacity to gain weight from birth to weaning (33 days) and had the largest adult size (weight). Family 35 was second to 13 in both these respects.

But that the differences we are now considering are qualities not wholly reflected in rate of gain or adult weight as indicated by the further fact that we have so far been able to draw no definite distinction between Families 13, 2, 32 and 39, while in Wright's measurements, 2, 32 and 39 were definitely inferior to 35. It thus appears certain that the present distinctions as above described rate Family 35 as one having a definite constitutional peculiarity. Each time that we have tried to place Families 2 and 32 with respect to these qualities we have freshened an impression that 32 was more like 35 and that 2 was more like 13. This might be of interest in connection with the similar but indefinite likenesses in the character of the local ulcerative processes were it certain that the judgment in the two cases was formed independently. It is, however, true that the facts with regard to the character of the lesions were well established first and were the stimulus to the effort to formulate the impressions as to general constitution. We must therefore rest the case for general constitutional attributes on the comparative merits of the two families, 35 and 13, which even though giving an incomplete account of the animal material is essentially satisfactory in that the differences there are well defined and they are clearly at the extremes so far as these groups of animals are concerned.

SUMMARY.

In summary the observational part of this paper may be restated as follows:

1. On intracutaneous inoculation with the tubercle bacillus inbred guinea pigs develop local lesions having familial characteristics.

Family 35 which has a high natural resistance against tuberculosis as measured by length of life after inoculation develops a compact nodule with a relatively restrained type of ulceration. Family 13 which has a low natural resistance against the disease develops a softer, less well demarcated, more edematous nodule with a much less restrained, more destructive type of ulceration. Differences of the same general order develop when local lesions are created on the skin with turpentine or cantharides in these two families. In other words, the distinctions relate to the general quality of inflammatory reactions in these animals rather than to a specific reactivity against the tubercle bacillus or its product.

Families 2 and 32 which are intermediate between 13 and 35 in natural resistance develop local lesions which are on the whole intermediate also, but more like those of Family 13. In so far as it is possible to assign closer relationships it would seem that Family 32 reacts somewhat more like Family 35 and Family 2 more like Family 13.

Family 39, the lowest in natural resistance of the families available for this study presents a peculiar type of local lesion which begins with a compact nodule not to be distinguished from that of the most resistant family but often ends with an extensive indolent, excoriating, crusted ulceration. This type of lesion is not always presented by this family but has never been encountered in a very extended experience with the other families.

In the case of Family 13, but in no other, the character of the local lesion finds frequent expression in the reactions of the adjacent lymphatics and lymphatic nodes. Fresh nodules and small ulcers frequently appear along the line of the lymphatics. The lymph nodes frequently become adherent to the skin, ulcerate through and present residual persistent sinuses.

2. Families 35 and 13 present differences in the quality of their

tissues suggestive of variation in constitutional character, as observed in connection with a minor surgical operation. They also appear to differ somewhat in general conformation, muscular tone and in nervous irritability or temperament.

Interpretation.

A. Present Observations as Related to the Observed Differences in Resistance against Experimental Tuberculosis.

Our conception of disease rests on the fundamental proposition that absence of injury to either structure or function is health, and similarly when disease processes are compared, that the lesser the injury or the greater the tendency of any injury to repair the greater the approach is to health, the more favorable or inclinable to health is the condition. And in connection with local insults to tissues of all sorts age old surgical experience has crystallized into certain judgments the background for which cannot be materially reinforced by direct experiment. They are essentially axiomatic. Thus a surgeon recognizes as a healthy ulcer one which is under all the circumstances making an unmistakable active progress toward the healed condition.

It seems therefore entirely justifiable to consider that in the present experiments the observations on the local lesions exhibit a considerable, perhaps an unexpectedly considerable correlation with the previously determined general resistance of the families of animals against tuberculosis. Thus Family 39 showing by all odds the least general resistance develops a local lesion which in its later phases shows neither the tendency to heal nor any appreciable measure of the activity which experience teaches must be associated with the development of such a tendency. All of the lesions in all of the other families have shown activities which if fully developed would eventually be expected to result in a healed lesion.

Family 13, next in order of increasing resistance, and in fact all of the other families exhibit a good measure of the kind of activity usually considered essential to the establishment of a healing process. But in Family 13 this is associated most definitely with an interfering, more or less progressively destructive process which cannot at present be segregated for further description but which finds its expression in

the larger, more tumid type of lesion, and particularly in the way in which the ulcer follows out to the margin of the inflammatory area.

Family 35 exhibits an active process in which this destructive tendency is under most restraint. This family also shows the largest percentage of actually healed lesions at most times.

Families 2 and 32 exhibit active lesions on the whole less clearly defined but probably intermediate in place between 35 and 13. Based on the percentage of healed lesions eventually resulting, it appears proper to say that 32 is more like 35 and 2 more like 13.

The sum total of these relationships may be expressed in the following arrangement of family numbers in order respectively of decreasing general resistance and of decreasing reactive quality. The less definite likenesses are suggested by the brackets.

General resistance: 35, 2, 32, 13, 39.
Inflammatory reactivity: 35, 32, 2, 13, 39.

With reference to our problem of locating reactive characteristics which are both based on the inheritance and capable of accounting for differences in resistance the agreement is considerable. It seems justifiable to conclude that the variations in inflammatory character partly account for the observed differences in general resistance. The fact that Families 2 and 32 do not follow the scheme makes it clear, on the other hand, that resistance is not wholly determined by inflammatory character.

B. General Considerations with Reference to Immunity against Tuberculosis.

The immunity reactions and relationships in tuberculosis are much less well understood than is the case in many other diseases. It has been the general opinion that the humoral and cellular constituents of the blood are of relatively less significance. Much weight is laid on the tuberculin reaction and the hypersensitive state of which it is the available index. There is current a suggestion that possibly immunity in this case rests largely on some peculiar relation of the tissues to the infection and the idea is summed up in the term "tissue resistance." For the most part attempts at a better definition of this

idea experimentally have resolved themselves into a study and interpretation of one or another form of the tuberculin reaction.

Analysis of the problem of tissue resistance shows that the epithelial tissues may be left out of consideration in this instance as they are but secondarily involved in the disease. The unformed constituents of the body fluids and the polymorphonuclear series of leucocytes are not to be neglected but are outside the present argument. There remain the fixed connective tissues, the blood vessels, particularly the capillaries, and the reticulo-endothelial cell system inclusive of the phagocytic macrophages and the blood cells of the mononuclear series.

The fixed connective tissues might be thought of as the point of primary injury and they certainly appear frequently in the reparative and healing process. Lewis and Newcomer (6) attempted to show some relationship between the individual capacity of the normal rabbit to form connective tissue in response to trauma and the relative susceptibility to inoculation tuberculosis of the same animal at a slightly later period. There was no such relationship shown in the experiments. The subject is difficult of approach and the negative result of one type of experiment does not exhaust it.

The other elements involved, through the hypertrophic reactions of the blood vessels give character to the process as a granuloma and by means of the temporary organization of the other cells create the typical tubercle as a second characteristic anatomical feature of the disease. It is probably not going too far to assume that factors which determine the qualities of this imperfect and more or less transient organization of the mesothelial tissues are also likely to be factors in the resistance to the progress of tuberculosis after its establishment and less tangibly perhaps in any immunity which may be naturally possessed or acquired. In this limited sense our observations appear to be in accord with the idea that tissue resistance may be a nonspecific factor of import to the natural immunity to tuberculosis. Only further study can show how far, if at all, these processes can be intrinsically modified by the acquisition of a specific immunity.

C. Genetic Considerations.

From the genetic standpoint it seems necessary to assume that we are dealing here with qualities that rest on a blending type of inherit-

ance. The reaction characters themselves merge one into the other so that it is only on the average or in extreme and therefore type cases that the distinctions can be clearly seen. Moreover, in so far as we have dealt with crossbred animals, the distinctions have been blurred and appear wholly as individual variations.

The view is held today by specialists in the field that blended inheritance is a consequence of the interaction of multiple genetic factors. This would agree with an *a priori* conception of the case since it is difficult to see how so complicated a process as an inflammatory reaction carried through from initial stages to healing could be under the control of a single or even of any very small number of functional or morphologic units. It is nevertheless possible to see in the results some suggestion of segregating unit characters. The peculiar qualities presented by the extreme cases in Family 39 are to the point. It would seem not unlikely that they rest on a recessive trait or traits. Otherwise they should occasionally be encountered in the crossbreds. Similarly it would appear that those cases in Family 13 in which the inflammation proceeds with least restraint and in which the lymphatic channels and nodes show lesions peculiar to the family, may well be evidence of action or failure to act in part controlled by a recessive character or characters since they have not been encountered in the crosses.

D. Constitutional Considerations.

Belonging essentially to the prebacteriologic era of pathology is the conception that susceptibility to infectious disease is more or less definitely related to fundamental inheritable qualities which find expression in physical conformation, that is, "physical type," and in peculiarities of function, that is "constitution." The terminology was on the whole very loose and interchangeably employed. Constitution also was often thought to be expressed in physical characteristics. When functional characteristics were thought of as directly related to disease the term "diathesis" was frequently used. Thus people of a certain inherited "constitution" were regarded as especially liable to tuberculosis, particularly to that of the lymphatic glands on the basis of a "scrofulous diathesis."

When ideas were crystallized during and after the classical bacteri-

ological studies of the latter quarter of the last century the conception of the scrofulous diathesis was first amplified in an attempt to harmonize it with new observations, and then almost if not quite discarded as being at best inadequately grounded. The considerations advanced in amplification of the conception are of considerable interest in the present connection.

It was first shown, of course, that the lymphatic lesions characteristically associated with the diathesis were tuberculous and that they had in general the same etiology as pulmonary tuberculosis. It was soon very evident that it was difficult if not impossible to discriminate between those physical characteristics which might be preexistent and possibly reflect predisposing causes, and those which were the consequences of long continued chronic disease transmitted by contact infection from generation to generation and often persisting in the individual from earliest childhood to old age. It also appeared that many of the other lesions particularly those of the skin which had frequently been regarded as evidences of a scrofulous diathesis were not tuberculous but were due to casual infection with staphylococci, streptococci and probably other microorganisms.

This recognition of many of the appearances as "consequences" greatly weakened the whole conception. The further evidence that if there was a constitutional predisposition it was not strictly specific for tuberculosis but involved other inflammatory processes as well put the question out of touch with the progressive thought of the time, which was primarily engaged in establishing specific relationships, either of etiology or immunity.

With the coincident and tremendous improvement in hygienic conditions and nutritional well being in Europe and especially in America, tuberculosis and the minor infections referred to have a greatly diminished prevalence. It is now to be accepted that practically all of the aforetime ability to segregate a type of people having the scrofulous diathesis (if such there are) was dependent on the continued manifestation of the infections to which they are susceptible.

We think it of interest and significance that this first experimental approach to the question with suitable material develops a picture which fits so well with the conception of a scrofulous diathesis as it stood at about the beginning of the present century. We have ob-

served an inherited group of reactive qualities which are related to susceptibility to tuberculosis and also find expression in the character of the tissue changes in tuberculosis and in some simple inflammatory reactions. Respecting the limitations imposed by species differences this would seem to be as close as it could be hoped to come to an experimental definition of the "scrofulous diathesis."* It seems possible that a study of skin reactions to various inflammatory irritants, especially such as could have no antigenic properties, would develop facts of interest in connection with more general studies on human constitution.

SUMMARY.

Five families of strictly inbred guinea pig whose general resistance to experimental tuberculosis had previously been determined by Wright and Lewis have now been studied with reference to the characteristics of the local lesion produced by intracutaneous inoculation with the tubercle bacillus.

It is found that there are clearly recognizable familial types based on the size and quality of the nodular lesion, the ulcerative lesion consecutive to this and the general effectiveness of the healing process when in evidence.

Family 39 which has the lowest general resistance forms an initial papule which does not differ appreciably from that formed by Family 35 which is the most resistant. In the ulcerative stage Family 39 shows an indolent excoriating process which exhibits none of the qualities which would be expected to make for healing.

Family 35 exhibits a firm primary papule and nodule followed by an active healthy type of ulceration which is definitely restrained in comparison with that of the low resistance Family 13. Family 35 also presents the largest number of completely healed lesions in the later observation periods.

* For a modern discussion of the whole question of constitution and physical type as related to susceptibility to particular diseases or disease groups, reference may be made to the recent monograph of Draper (7) and a still more recent essay by Stockard (8). These authors do not consider the scrofulous diathesis. The most recent extended review of the subject with which we are familiar is by Schluter (9).

Family 13 shows a larger, softer, primary papule and nodule than any of the others. The ulcer when formed is less restrained; that is, it is larger in proportion to the total size of the lesion. It is also more generally destructive. This family also shows a singular tendency to the formation of secondary ulcers along the lymphatic channels leading toward the adjacent lymph nodes. The adjacent lymph nodes are likewise more severely affected and frequently ulcerate through the skin giving rise to residual discharging sinuses.

Families 2 and 32 are less definitely characterized. They are of the same order as 35 and 13 in that the ulcerations are active and healthy. Such similarities as are recognizable place Family 2 more nearly with 13 and Family 32 more nearly with 35.

In general the qualities of lesion exhibited are such as to agree with the general resistance as previously determined and it is believed that the qualities underlying the tissue reaction may be safely considered to be among the influential factors in the make-up of the natural resistance against tuberculosis.

The differences in reaction are in part manifested against agents causing simple inflammation also, and hence must be designated as non-specific with reference to the tubercle bacillus.

There are definite indications of other "constitutional" differences in the character of the tissues and the general make-up of these families.

The accumulated evidence from the study of the separate families and intercrosses between them is to the effect that the differences in question are transmitted by the blending type of inheritance and are therefore controlled by multiple unit characters. It is also suggested by the observations that certain of the significant characters are recessive in nature.

It is of considerable interest that the observations agree very well with the older conception of an inherited, predisposing, constitutional diathesis as a significant factor in the incidence of tuberculosis.

BIBLIOGRAPHY.

1. Wright, S., and Lewis, P. A., *Am. Naturalist*, 1921, lv, 20.
2. Lewis, P. A., and Loomis, D., *J. Exp. Med.*, 1924, xl, 503.
3. Lewis, P. A., and Loomis, D., *J. Exp. Med.*, 1925, xli, 327.

4. Lewis, P. A., and Loomis, D., *J. Exp. Med.*, 1926, xliii, 263.
5. Lewis, P. A., and Loomis, D., *J. Exp. Med.*, 1928, xlvii, 437.
6. Lewis, P. A., and Newcomer, H. S., *J. Exp. Med.*, 1919, xxix, 369.
7. Draper, G., Human constitution, Philadelphia and London, 1924.
8. Stockard, C. R., *Medicine*, 1926, v, 105.
9. Schluter, R., Die Anlage zur Tuberkulose, Leipsic and Vienna, 1905.

EXPLANATION OF PLATE 23.

FIG. 1. Typical lesion on Family 35.

FIG. 2. Typical lesion on Family 13.

FIG. 3. Typical lesion on Family 39.

The animals were from a series inoculated on the same day, intracutaneously, with 1/10 mg. of a culture of bovine type of tubercle bacillus. The drawings are natural size and were made on the 20th day after inoculation.

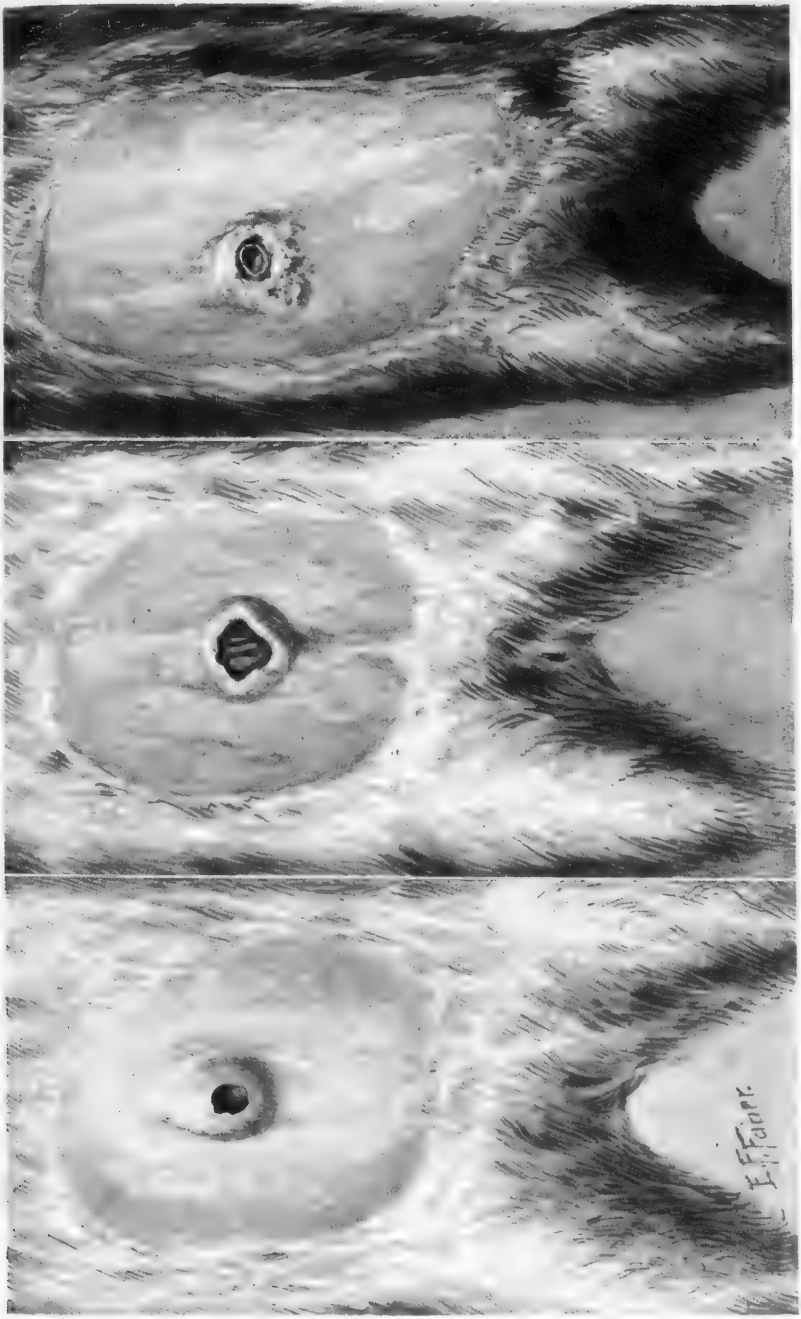

FIG. 3.

FIG. 2.

FIG. 1.

(Lewis and Loomis: Ulcerative types and inheritance.)

THE ANTIGENIC COMPLEX OF STREPTOCOCCUS HÆMOLYTICUS.

II. Chemical and Immunological Properties of the Protein Fractions.

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The presence of several antigenic substances in the hemolytic streptococcus has been indicated previously; two of these were non-type-specific, while a third exhibited type specificity. Of the non-type-specific substances one was species-specific and seemed to be non-protein (1); the other, a widely reactive "nucleoprotein" fraction, was an antigenic protein and showed partial cross-reactions with similar proteins from related species of Gram-positive cocci (2). The type-specific substance was demonstrated in extracts of hemolytic streptococci by the use of sera absorbed with intact bacteria of heterologous hemolytic streptococci (3). By this means the non-type-specific antibodies were removed from the sera and the remaining type-specific antibodies were detected with the precipitin test.

This report is concerned with the chemical and immunological relationships of the type-specific substance and with a review of the properties of the group reactive nucleoprotein for purposes of comparison.

Since it was highly desirable to determine the nature of the type-specific substance, designated as M, attempts were made to isolate and purify it. With sera absorbed as described, several kinds of extracts were tested for the presence of the type-specific substance. It was found that dried bacteria pulverized in a ball mill, and extracted with N/100 NaOH in the cold, yielded some type-specific substance, M, although the non-type-specific nucleoprotein, P, predominated. Similar extraction with 0.85 per cent NaCl, instead of N/100 NaOH,

yielded a somewhat greater proportion of M than of P. However, the separation of the two substances from each other was too difficult to make either of these extracts a profitable source of M. HCl extracts, as previously described (3), yielded the largest amount of M and the smallest of P. While it was obvious that a procedure which involved heating in a boiling water bath for 15 minutes with N/20 HCl was a dubious method of obtaining material for chemical analysis, it was hoped that some idea of the nature of the type-specific substance might be gained and thus better methods of extraction be devised. The HCl extracts, therefore, were the main source of M. N/20 to N/40 HCl gave maximum yields of M, while N/1 HCl destroyed all the serologically active materials, and dilutions of N/80 and greater yielded only small amounts of M.

Methods.

Antibacterial sera were prepared by intravenous injections first of killed then of living cultures. The plan of immunization was a series of four daily injections followed by a rest period of 3 days. The first series consisted of four daily injections of 1 cc. of heat-killed culture, the second series consisted of 2 cc. doses, the third of 0.5 cc. doses of living culture, the fourth of 1 cc. doses of living culture the fifth of 2 cc. doses of living culture. If the precipitin titer of the test bleedings, taken 7 days after the last injection, was unsatisfactory, another series of three injections of 5 cc. each of living culture was given. This last series was repeated until a satisfactory titer was obtained, or until the animal was discarded if the immune response continued inadequate. Sometimes animals which did not yield satisfactory sera by this method were given a rest period of 6 weeks to 2 months. They were then further immunized either with one series of 2 cc. doses of killed culture followed by series of 5 cc. doses of living culture as before, or else with series of 10 cc. to 50 cc. of killed culture concentrated to 2 cc. volume.

It was usually not easy to produce antisera of sufficient potency to give satisfactory immune precipitates with the type-specific substance. Some rabbits yielded potent antisera in 6 weeks, while others of the same group of animals responded only moderately. Often it was necessary to continue immunization for several months before obtaining antisera of the high titer necessary for work with the precipitin test. Usually a high agglutinin titer was reached without difficulty. This may depend on the fact that small amounts of antibody in the serum are more readily detected by agglutination than by precipitation, while a much higher concentration of antibody is necessary if the object is the detection of small amounts of antigen. Since the latter is the case in the study of substances obtained from bacteria, a potent agglutinating serum is not sufficient but a pre-

cipitating serum of high titer must be obtained even though considerable time is required for its production.

The following was the usual method of preparing extracts of the type-specific substance. The bacteria from 18 liters of plain broth culture were suspended in 200 cc. of hot N/20 HCl, made by diluting N/1 HCl with 0.85 per cent NaCl solution, and heated for 10 minutes in a boiling water bath, then cooled and centrifuged. After removal of the supernatant fluid, a second extraction was made similarly with 200 cc. of fresh solvent. In all, six extractions were made before the bacterial residue was discarded. The volume was usually decreased to 100 cc. and then to 50 cc. for the later extractions. Preliminary experiments showed that, within reasonable limits, the yield of M was independent of the volume. The first two or three extracts contained the majority of M; but sufficient amounts were still present in the later extracts to make their preparation worth while. The supernatant fluids were combined and neutralized with N/1 NaOH. The precipitate which formed on neutralization was inactive serologically, hence was discarded. Crystals of sodium acetate (about 20 gm. per liter) were added to the water-clear, slightly yellowish supernatant fluid together with 3 or 4 volumes of 95 per cent alcohol. Most of the M was brought down in the resulting precipitate, while the non-type-specific C substance, probably carbohydrate in nature, was largely left in the supernatant fluid. A few reprecipitations with alcohol served to separate this material quite completely from the type-specific M. After standing in the ice box overnight, the alcoholic precipitate was removed by centrifugation and the supernatant fluid was saved for purfication of the non-type-specific C. The precipitate was freed from alcohol by evaporation at 55°C. and then redissolved in 15 cc. or 20 cc. of saline; it was then centrifuged. Although most of the alcoholic precipitate was insoluble, precipitin tests showed very little less M in solution than there was in the original extract. Washings from the insoluble precipitate were added to the main solution and the insoluble part finally discarded. The M substance was reprecipitated with three volumes of 95 per cent alcohol and redissolved in a smaller volume of salt solution than before. This process was repeated five or six times. Crystals of sodium acetate were added at alternate precipitations to insure the presence of electrolytes. Only a small fraction of the second alcoholic precipitate was insoluble in salt solution; and after one or two more alcoholic precipitations all of the precipitate was readily soluble in salt solution. Further purification was effected by precipitating with a few drops of 10 per cent acetic acid. The precipitate was redissolved in salt solution by the addition of a drop of NaOH, and the process repeated several times in spite of some loss of active material. Micro-Kjeldahl determinations on the acetic acid supernatant fluids showed some purification since the removal of nitrogenous material was greater than the removal of M. After three reprecipitations with acetic acid, the precipitate was washed three times with distilled water. The test for chlorides was negative in the second and third wash waters. The precipitate was then dehydrated by washing three times with redistilled alcohol, and finally three times with

redistilled acetone. It was then filtered on hard filter paper and dried in a desiccator. The yield from 45 liters of broth culture was, in one instance, 18 mg. of a fine white powder.

The properties of the 18 mg. lot of M were as follows:[1]

1. It gave a precipitate with copper sulfate.
2. It gave a violet biuret test which was not very strong.
3. With Millon's reagent it gave a white precipitate which remained on heating and did not become colored. This precipitate was not crystalline.
4. With the xanthoproteic test it gave a faint color on heating with HNO_3 which deepened slightly on the addition of NH_4OH.
5. The Molisch test was positive but not strong.
6. The percentage of total nitrogen was 14.6; while only a trace of amino nitrogen was present, too small a quantity to determine.
7. The acid equivalent was 552.

Earlier tests on less purified material showed that M withstood autoclaving for 20 minutes at 15 pounds pressure when the solution was neutral or acid (N/20 HCl), but not when the reaction was alkaline (N/20 NaOH).

Experiment 1.—Precipitin tests with the purified material were made against a number of homologous and of heterologous antibacterial sera to test its specificity. These results are recorded in Table I.

In all experiments, the final volume of precipitin tests was 0.5 cc. Each tube contained 0.1 cc. of serum. Controls of antigens alone and of sera alone were uniformly negative. The tests were read after 2 hours at 37°C. and again after standing overnight in the ice box. The overnight readings are recorded in all tables.

The extract still reacted with two homologous sera at a dilution of 1–300,000. With four heterologous sera, it was negative in all dilutions; with three heterologous sera, it gave weak reactions; and with one heterologous serum, it gave strong cross-reactions. These cross-reactions were not due to the C substance, for they were not observed with Serum Q696, known to be very potent in C antibodies; and, as will be shown later, the cross-reactions were not present after tryptic

[1] The author is greatly indebted to Drs. Heidelberger and Goebel for this analysis and for several others given in this paper, as well as for much helpful advice in the chemical procedure.

or peptic digestion of this extract. This cross-reaction was, therefore, due to a protein. Since this extract reacted very slightly, if at all, with potent anti-P sera, it seemed doubtful whether P impurities were responsible for the cross-reactions. Later work with the anaphylactic reaction definitely eliminated P as the cause of this non-type-

TABLE I.

Precipitin Reactions.

Purified Type-Specific M Substance Prepared from HCl Extract.

Serum			Antigen. HCl extract from Strain S43, *Type* S60. Final dilutions 1:		
No.	Prepared against		19,000	75,000	300,000
	Strain	Homologous type			
Q868	S60	S60	+++	++	+
Q865	"	"	+++	++	+
		Heterologous type			
Q311	S23	S23	−	−	−
Q611	"	"	+	+	±
Q612	"	"	+	0	0
Q613	"	"	−	−	−
Q244	"	"	−	±	−
Q245	"	"	−	−	−
R266	S39	"	++*	++±	+
Q696	New York 5	Scarlatinal	−	−	−
Normal			−	−	−

*Test made at a different time: dilutions only estimates.

In all tables ±, +, ++, +++, ++++ indicate degrees of precipitation; − indicates a negative reaction; 0 indicates that test was not made.

specific reaction and necessitated the assumption of another non-type-specific protein in the antigenic complex of the hemolytic streptococcus; this fraction was present only in certain strains, and antibodies for it were only found in occasional antibacterial sera. The anaphylactic reactions which confirmed the existence of this somewhat hypothetical substance, designated as Y, are given in a subsequent paper.

In spite of the evident fact that the HCl extract was not entirely freed of non-type-specific fractions by the methods used for its purification, certain information in regard to its nature was acquired by the study of the material in this state. During the preparation and early tests it had become apparent that the type-specific substance behaved more like a protein than a carbohydrate: it was precipitated by the ordinary protein precipitants, including picric acid; and preparations, purified as described, contained 14 per cent nitrogen. The effect of removal of the NH_2 group on the serological specificity of M was, therefore, determined by treatment with nitrous acid.

TABLE II.

Precipitin Reactions.

Effect of Removing NH_2 Group from Purified Type-Specific M Substance.

Serum:* Q868, against Strain S60, *Type* S60		
Antigen: HCl extract of Strain S43, *Type* S60		
Final dilutions 1:	Treated with HNO_2 for 1 hr.	Blank control not treated with HNO_2
13,500	++	++++
27,000	+±	++++
54,000	+	+++
108,000	±	+±
216,000	±	+

* 0.1 cc. in each tube.

The same series of antigen dilutions tested against normal rabbit serum was completely negative.

Experiment 2.—Samples of "purified" M were treated with nitrous acid for 10 minutes and for 1 hour. After neutralization they were tested for M by means of the precipitin reaction.

Such treatment for 10 minutes caused a considerable reduction in the amount of precipitate with immune serum, and treatment for 1 hour reduced the amount very greatly, as shown in Table II. This experiment gave considerable indication that the type-specific M was a protein.

The effect of tryptic and of peptic digestion was also determined. A typical result is given in Experiment 3.

Experiment 3.—In this experiment, 2 per cent trypsin (Fairchild's) was allowed to act for 18 hours at 37°C., on concentrated M, in a solution sufficiently alkaline to give a permanent pink color with phenolphthalein. A control was included with the same amount of enzyme previously inactivated by heating in a boiling water bath for 10 minutes. Toluene was used as a preservative. After the incubation period, the digest and its control were neutralized with HCl and then heated for 10 minutes in a boiling water bath.

A similar digestion experiment was performed with pepsin in solutions made sufficiently acid with HCl to turn Congo red paper blue. After incubation, the digestion mixtures were neutralized with NaOH and heated to destroy the enzyme. Precipitin tests with these digests and with their controls are recorded in Table III.

TABLE III.

Precipitin Reactions.

Effect of Tryptic and of Peptic Digestion on the Type-Specific M Substance.

Serum:* Q868, against Strain S60, of homologous *Type* S60

Antigen: HCl extract of Strain S43, *Type* S60

Cc.**	Tryptic digest	Control with heated trypsin	Peptic digest	Control with heated pepsin
0.05	−	++±	−	++±
0.025	−	+++	−	+++
0.012	−	+++±	−	+++±
0.006	−	+++	−	+++
0.003	−	++	−	++
0.0015	−	++	−	+±

* 0.1 cc. in each tube.

** Dilution of antigen unknown.

Table III shows that digestion of M with either pepsin or trypsin completely destroyed its ability to form precipitates with homologous antibacterial sera. Later it was determined that 0.5 per cent trypsin acting for 10 to 20 minutes was sufficient to destroy this antigen.

Seibert (4) in her studies on the chemical composition of the active principle of tuberculin, states that trypsin in neutral solution does not digest whole protein readily, although it does digest some of the split products at this pH. Following this suggestion, tryptic digestion of the M substance was carried out at pH 7.0. Digestion at this reaction was, however, just as complete as it was at an alkaline reaction.

It was also shown that the negative precipitin reactions after digestion were not due to the possible inhibiting action of digestion products since the peptic digest added to undigested M did not change the range or the degree of precipitin reactions.

The digests used in Experiment 3 were also tested against the heterologous serum which gave the best cross-precipitations. Table IV shows these tests. This non-type-specific substance also was destroyed by both tryptic and peptic digestion: the digests no longer reacted with this serum, although the controls with inactivated

TABLE IV.

Precipitin Reactions.

Effect of Tryptic and of Peptic Digestion on the Non-Type-Specific Substance in Purified HCl Extracts.

Serum:* R266, against Strain S39, of heterologous *Type* S23

Antigen: HCl extract of Strain S43, *Type* S60

Cc.**	Tryptic digest	Control with heated trypsin	Peptic digest	Control with heated pepsin
0.05	−	+±	0	+±
0.025	0	0	−	++±
0.012	−	+++	0	++±
0.006	0	0	−	++
0.003	−	++±	0	0

* 0.1 cc. in each tube.

** Dilution of antigen unknown. The antigen preparation in this table is the same as that used in Table III.

enzyme gave good cross-precipitations. Evidently the non-type-specific substance concerned here was a protein.

Numerous tests were made with regard to the time and the concentration of enzyme necessary for digestion of these two substances. It was observed consistently during a large series of such experiments that the non-type-specific fraction was digested much more rapidly than the type-specific. A separation of these substances on this basis was attempted, but the loss of the type-specific fraction was too great to make such a method useful.

Since the digestion experiments, supported by the nitrous acid test,

indicated clearly that the type-specific M was a protein, attempts were made to separate it from its impurities by salting out, not only from HCl extracts but also from NaOH and NaCl extracts of pulverized bacteria. Ammonium sulfate was found more satisfactory than sodium sulfate for this purpose; and a certain amount of separation of the type-specific substance from the non-type-specific could be attained. The non-type-specific substance was precipitated more readily than the type-specific by the lower concentrations of the salt; but in order to remove most of the non-type-specific materials a great loss of the type-specific substance was also sustained, although full saturation with ammonium sulfate was necessary to precipitate all of the M. This method was therefore abandoned. Purification by adsorption on voluminous alumina and on kaolin was also attempted without much success.

Finally, all available material was used in immunization and in anaphylaxis experiments, and further attempts to isolate M in a pure form were suspended until more material could be collected for this purpose. The immunization experiments follow.

Experiment 4.—Four rabbits were injected with HCl extracts from two strains of different types; two received intravenous injections of extracts from each strain. Five daily injections were given followed by a rest of 5 days. The dose was then doubled for the next five daily injections. This plan was followed for four series of injections. Test bleedings taken after the second and fourth series showed no agglutinins and no precipitins for the type-specific M or for the non-type-specific P and C substances. One rabbit from each series was then given a rest and the two remaining animals continued with all of the available material. Each received the HCl extract from 9 liters of plain broth culture in six daily injections in each of the two succeeding series of injections. At the end of this time these two animals had been in process of immunization for 10 weeks. Test bleedings after the fifth and sixth series of injections still showed no trace of antibodies. All four animals were finally given intravenous injections of whole bacteria in the same manner ordinarily employed for producing antibacterial sera. All responded promptly with the production of antibodies at the usual time after such injections. The failure to produce agglutinins or precipitins in response to the HCl extracts was not, therefore, due to inability of these rabbits to produce antibodies.

The failure to give an immune response to intravenous injections of HCl extracts could not be considered final proof that the M substance was not antigenic. The explanation might be that the rabbits had

not received enough antigen, or that the protein had been so altered by extraction with heat and hydrochloric acid that it could no longer function as an antigen. The latter hypothesis was tested as follows.

Experiment 5.—Two healthy young rabbits, weighing 1,800 gm. each, were immunized with a saline extract from Strain S43. This strain was selected because it gave a uniformly large yield of M. To the dried and pulverized bacteria 0.85 per cent NaCl solution was added in the cold; and the extract was made by shaking in the cold overnight. The smallest amount of 10 per cent acetic acid necessary to give maximum precipitation was added in the cold, and the extract again left in the ice box overnight. The precipitate, removed by centrifuging, was dissolved in a suitable small volume of saline with the aid of a few drops of N/4 NaOH. With precipitin reactions as previously described (3), the presence of M in such solutions was proved. It was similarly shown that practically all of the M had been precipitated from the original solution with the acetic acid. The object of this precipitation with acetic acid was to concentrate the M sufficiently for intravenous injections with the least harmful chemical manipulation.

Each rabbit was given such an extract from 1.5 liters of original broth culture in four daily intravenous injections. After 5 days rest, double that amount was given in three daily injections. Test bleedings 5 days later showed the presence of P antibodies but no M antibodies. One of the animals was sick and died as a result of *B. lepisepticus* infection after two more injections. The remaining animal received three more similar series of injections. The extract from 3 liters of culture was used for the third series, the extract from 19.5 liters for the fourth series, and that from 15 liters for the fifth series. In spite of the fact that this animal received the extract from a total of 42 liters of broth culture over a period of 5 weeks, and that bleedings after the fourth and fifth series of injections showed a high titer of P antibodies, still there were no traces of M antibodies The anaphylactic test, reported in a succeeding paper, also failed to reveal the presence of M antibodies in the serum of this rabbit.

The failure to produce antibodies in these two rabbits by the injection of a simple saline extract gave weight to the previous evidence of the non-antigenicity of M which was obtained by the failure to immunize the four rabbits in Experiment 4 with HCl extract. Thus, although the type-specific substance in the hemolytic streptococcus is a protein, nevertheless, after it has been separated from the bacterial cell, it either does not give rise to antibodies when injected into rabbits intravenously, or else may do so only under conditions not yet determined. This substance seems, therefore, to be a haptene rather than a true antigen.

Solutions of the nucleoprotein P, on the contrary, are true antigens in that rabbits are easily immunized with them. Antisera from these animals are precipitated equally well by nucleoproteins from all strains of hemolytic streptococci. This has been reported previously (2) and has been repeated and confirmed in the present experiments. Nucleoproteins from non-hemolytic streptococci and from pneumococcus also precipitate these hemolytic streptococcus anti-P sera to a certain extent. This fraction of the hemolytic streptococcus is, therefore, not only not type-specific but not even species-specific. It is possible that with appropriate methods this nucleoprotein portion could be further fractionated into a strictly species-specific part and into other parts which are responsible for the cross-reactions with other species. Such methods are not at present available.

The species-specific substance first described by Hitchcock is considered in the succeeding paper, in which there is also included a discussion of all these reactive substances.

SUMMARY.

The chemical and immunological characteristics of the type-specific substance (M) of *Streptococcus hæmolyticus* are considered.

1. A summary of the evidence for the protein nature of this substance follows:

(*a*) It is precipiated by the usual protein precipitants such as, dilute alcohol, dilute acetic acid, and picric acid.

(*b*) It contains 14 per cent protein nitrogen after considerable purification.

(*c*) It is progressively destroyed by removal of the NH_2 group by treatment with nitrous acid.

(*d*) It is completely and readily digested by trypsin and by pepsin.

2. "Purified" extracts react in relatively high dilution with homologous antibacterial sera, but do not precipitate most heterologous antibacterial sera or sera potent in non-type-specific antibodies for the group reactive nucleoprotein P or for the species-specific probable carbohydrate C. Attempts to immunize rabbits with the type-specific protein have been unsuccessful, with simple salt solution extracts of

streptococci as well as with purified solutions. This protein seems, therefore, to have the characteristics of a haptene.

The type-specific substance (M) is contrasted with the so called nucleoprotein (P) which shows group relationships with nucleoproteins of related species and is the only fraction of hemolytic streptococcus extracts so far obtained which, after separation from the bacterial cell, is a true antigen leading to antibody production when injected into rabbits.

The occurrence of another non-type-specific protein (Y) is suggested by occasional cross-reactions of purified M with certain antibacterial sera. Since it has not been separated from extracts containing the type-specific M, little is known of it either chemically or serologically. The cross-reaction disappears on tryptic or peptic digestion of the extract. The fact that such extracts do not show cross-reactions with anti-P sera is evidence that this non-type-specific protein is not P.

BIBLIOGRAPHY.

1. Hitchcock, C. H., *J. Exp. Med.*, 1924, xl, 445.
2. Lancefield, R. C., *J. Exp. Med.*, 1925, xlii, 397.
3. Lancefield, R. C., *J. Exp. Med.*, 1928, xlvii, 91.
4. Seibert, F. B., *Am. Rev. Tuberc.*, 1926, xiii, 431.

THE ANTIGENIC COMPLEX OF STREPTOCOCCUS HÆMOLYTICUS.

III. Chemical and Immunological Properties of the Species-Specific Substance.

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In a previous paper the chemical and immunological relationships of the serologically active substances in extracts of hemolytic streptococci were discussed. The characteristics of the protein fractions, both type-specific and non-type-specific, were considered (1). The protein nature of the type-specific substance (M) was supported by its high nitrogen content (14 per cent), by its precipitation with protein precipitants, by its loss of activity on removal of the NH_2 group, and by its rapid and complete digestion with pepsin and with trypsin. Attempts to immunize rabbits with it after separation from the bacterial cell were unsuccessful. The so called nucleoprotein fraction (P) gave cross-reactions with nucleoproteins from related bacterial species and stimulated the production of antibodies in rabbits. The presence of another non-type-specific protein fraction (Y) was suggested by the cross-reactions of certain extracts consisting chiefly of the type-specific M. These cross-reactions disappeared on digestion with pepsin or with trypsin. This substance was, however, distinct from the nucleoprotein P since no cross-precipitation reactions were obtained with anti-P sera. It was not isolated but was only obtained in association with the type-specific protein.

The present paper is concerned with the species-specific substance, which Hitchcock first described as a "residue antigen" (2). This report also contains a discussion of the data concerning all these serologically active substances, including those described in the preceding paper (1).

It was desirable to collect and purify enough of the species-specific material, called the C substance, to determine its chemical nature. It exhibited an apparent chemical similarity to the specific carbohydrates of pneumococci (3), and, like these substances, it formed a typical disc precipitate with antibacterial sera; but, unlike the polysaccharides of pneumococcus and other bacteria studied from this point of view, it was not type-specific but formed precipitates with antibacterial sera made from hemolytic streptococci of all types. It was present in every kind of extract of hemolytic streptococcus. Since it was not precipitated to any extent by the addition of three or four volumes of 95 per cent alcohol or with acetic acid, the supernatant fluids of all extracts used for the preparation of the protein fractions (1) were saved for the isolation of C, which was separated as far as possible from the accompanying impurities.

Methods.

The antisera used in the following experiments were prepared as indicated in the preceding paper (1). While almost all antibacterial sera contained a moderate concentration of C antibodies, occasional sera from rabbits immunized for long periods were exceptionally potent. Intensive immunization seems required for the production of sera with a high titer of C antibodies. Hitchcock (2), who prepared numerous antisera of this sort, used very large doses of heat-killed culture in immunizing his rabbits.

The solutions which had been saved for the preparation of C were neutralized and concentrated to dryness, or to a small volume, *in vacuo* at a temperature not exceeding 37°C. After concentration enough distilled water was added to dissolve the salts; insoluble substances were discarded, and 3 volumes of 95 per cent alcohol were added to remove some of the protein. After standing overnight in the ice box, the precipitate was thrown down in the centrifuge and discarded. The supernatant fluid was again concentrated *in vacuo* and was dialyzed in a collodion sac against running water until most of the salts were removed. The C substance dialyzed through parchment membranes and also through collodion sacs which were impermeable to pneumococcus and to green streptococcus specific carbohydrates, but its passage was prevented by less permeable collodion sacs with the result that only an insignificant loss of active material was sustained in dialysis. When tests for chlorides were negative, the solution was again concentrated *in vacuo*, and sodium acetate in the proportion of 20 gm. per liter of solution together with 20 volumes of 95 per cent alcohol were added. This was left on ice overnight and the precipitate removed by centrifugation. The supernatant fluid, concentrated and tested with a potent anti-C serum, showed little C still present;

hence it was discarded. Lower concentrations of alcohol did not precipitate all of the C. The precipitate was freed of alcohol by evaporation and taken up in 16 cc. of distilled water; 0.6 cc. of 1:1 HCl was added; and a heavy precipitate, formed in the cold, was removed by centrifuging. The supernatant fluid was made strongly alkaline with NaOH and the gelatinous precipitate which formed was removed. Since precipitin tests showed that neither the acid nor the alkaline precipitates contained much C, they were discarded. A second 20 volume alcoholic precipitate was made, followed by precipitations with acid and alkali as before; the acid and alkaline precipitates were discarded. This process was repeated four or five times until the addition of acid and of alkali no longer produced a precipitate; the solution was then neutralized and 2 volumes of 95 per cent alcohol were added. A gummy, deep yellow precipitate separated out, leaving only a small proportion of coloring matter in the supernatant fluid, from which, after concentration, a second 2 volume alcoholic precipitate removed still more gummy, yellow material. The two precipitates were combined, redissolved, and reprecipitated with 2 volumes of alcohol. Since the resulting precipitate did not contain much C, it was discarded. The C was precipitated from the combined supernatant fluids by adding 20 volumes of alcohol; it was redissolved in distilled water and further purified by another removal of gummy, yellow substances by precipitation with 2 volumes of alcohol. This process was repeated until no more precipitate resulted after the fourth addition of 2 volumes of alcohol. The reaction of the solution was adjusted to pH 9, and absolute alcohol was added until a precipitate began to form. After standing overnight in the ice box, this precipitate was removed and was found to contain all of the C. After two such precipitations, the precipitate was dissolved in a small volume of distilled water, made acid by the addition of a drop of 1:1 HCl, and dialyzed against running tap water for 48 hours and then against frequent changes of distilled water for 48 hours more. The solution was then concentrated *in vacuo* and thrown into a large volume of acetone. The resulting precipitate was dried and stored in a vacuum desiccator.

Since the yield after so much manipulation was very small, the chemical data are meager. The final product was a fluffy white powder, which readily yielded a colorless solution in distilled water. The ordinary protein color tests with such solutions were negative, and the Molisch reaction was positive to the limit of the titer in the precipitin test. The optical rotation of one preparation (from Strain S23) before the final purification was $-33°$. After the removal of additional impurities, the optical rotation was the same. This indicated that the impurities removed were optically inactive and that the true figure for the active material was very much greater than the one obtained. Since the final yield from this lot was only 5 mg., no further determination of the optical rotation was made. The micro-Kjeldahl and the

quantitative carbohydrate determinations for this preparation were also unsatisfactory on account of the small amount of material. For another lot (from Strain S43) with a yield of 20 mg., the micro-Kjeldahl showed 4.2 per cent nitrogen and the carbohydrate determination showed on hydrolysis 28 per cent reducing sugar, calculated as glucose. Whether this large amount of nitrogen was an impurity or a part of the C substance cannot be stated without more material. It is interesting to note in this connection that the Type I pneumococcus specific substance has 4 to 5 per cent of nitrogen, apparently as an essential

TABLE I.

Precipitin Reactions.

Non-Type-Specific Reactions of the Purified C Substance, the Probable Carbohydrate.

Antigen (tested against) serum		Antigen (tested against) serum	
C from Strain S43, *Type* S60. Final dilutions	R446 against Strain S3, *Type* S3. 0.1 cc.	C from Strain S23, *Type* S23. Final dilutions	R446 against Strain S3, *Type* S3. 0.1 cc.
8,000	+±	10,000	+++±
16,000	++±	20,000	++++
32,000	+++	40,000	+++±
64,000	+++±	80,000	+++±
128,000	+++±	160,000	+++
256,000	+++	320,000	++±
512,000	++	640,000	+±
1,024,000	+	1,280,000	+
2,048,000	±	2,560,000	±
		5,120,000	−

component of the specific polysaccharide, and only 28 to 31 per cent of reducing sugars on acid hydrolysis (4). While this similarity to the figures obtained for a single small preparation of C from hemolytic streptococcus may not be significant, it is noteworthy that a specific polysaccharide of this composition is already known. These two C preparations were used in the anaphylaxis experiments to be reported in a succeeding paper and are discussed again in that connection. Their precipitin reactions are given in Table I.

Experiment 1.—A series of dilutions of each of the C substances described above was tested by means of the precipitin reaction against an antibacterial serum especially potent in C antibody, as shown in Table I.

The immune precipitates obtained with these non-type-specific C substances and antibacterial sera were always typical discs, such as have been described for the type-specific carbohydrates of other species (5). The serum used in this experiment had an especially high titer of C antibodies as shown by the good precipitates which both C preparations gave in dilutions of 1:1,000,000 with stronger reactions in higher concentrations of C. As usual with these substances, a marked prozone was present, so that still greater concentrations of C gave only negative results. The cross-reactions within the species of hemolytic streptococcus obtained by Hitchcock (2) were confirmed in the present experiment, as shown by the cross-reactions of three distinct types of hemolytic streptococci: the C substances were derived from *Types* S60 and S23, and the serum was prepared against a *Type* S3 strain. It is unnecessary to record the numerous precipitin tests performed with other C preparations and other antisera, since this experiment is typical of the non-type-specific C reactions.

The C substance did not precipitate sera prepared against the other commonly reactive fraction, the nucleoprotein P.

The effect of tryptic and of peptic digestion on the C substance was determined since these enzymes digested the type-specific M so easily. Experiment 2 was carried out for this purpose.

Experiment 2.—Fairchild's pepsin and trypsin were made up in 4 per cent solutions, the pepsin in salt solution and the trypsin in phosphate buffers. The pepsin was made sufficiently acid with HCl to turn Congo red paper blue, and the trypsin was made just alkaline to phenolphthalein with NaOH. Samples of each were heated for 10 minutes in a boiling water bath for use with the controls. A highly active C solution of undetermined concentration from Strain S23, *Type* S23, was mixed in equal parts with active and with heated pepsin and with active and with heated trypsin and incubated at 37°C. for 3 days with toluene as a preservative. The reaction was kept approximately constant throughout the experiment. Precipitin tests made daily showed that no change in titer had occurred by the end of the 3rd day; hence concentrated M extract was added in order to test the activity of the enzymes. The mixtures containing active enzyme were divided into two equal parts, and one part was heated for 10 minutes in a boiling water bath to inactivate the enzyme. Equivalent amounts of M extract from Strain S43, *Type* S60, were added to each tube, and the incubation at 37°C. continued. After 24 hours the digestion of M with trypsin was not quite complete, but digestion with pepsin was complete. The peptic digestion was therefore terminated by heating the mixtures in a boiling water bath for 10 minutes; while the tryptic

digestion was continued for another 24 hours, at which time the precipitin test showed that digestion of M was practically complete and this digestion was also terminated by inactivating the enzyme with heat. The total time of exposure of the C substance to active pepsin was 4 days, to active trypsin 5 days. The solutions were neutralized, cleared by centrifugation, and tested by setting up precipitin tests with antibacterial sera, one of which was potent in M antibodies, the other in C antibodies. Table II shows these reactions.

TABLE II.

Precipitin Reactions.

Effect of Peptic and of Tryptic Digestion on the C Substance.

Antigen: Digestion mixtures consisting of C from Strain S 23, *Type* S 23, and M from Strain S 43, *Type* S 60

Antigen dilutions 1:	Anti-C serum† (to test digestion of C)		Anti-M serum‡ (to test digestion of M)	
	Peptic digest	Undigested control with heated pepsin	Peptic digest	Undigested control with heated pepsin
2	+*	+*	−	+++**
4	+±	+±	−	+++±
8	++	++	−	+++
16	++±	++±	−	++±
32	++	++	−	++
	Tryptic digest	Undigested control with heated trypsin	Tryptic digest	Undigested control with heated trypsin
2	+*	+*	+	+++**
10	++	++	+±	+++
40	++	++	+±	++
160	+	+	+	+

† Serum Q696 was against Strain New York 5, one of Dr. Dochez' scarlatinal strains, and was potent in non-type-specific C antibody.

‡ Serum Q868 was against Strain S60, *Type* S60, and was potent in M antibody for that type, but lacking C antibody.

*These were disc precipitates, characteristic of C reactions.

** These were flocculent precipitates, characteristic of M reactions.

At the end of the digestion experiment, all tubes contained mixtures of M with C, since the former was added to control the activity of the enzymes during their contact with C. All mixtures were tested against both kinds of antiserum in order to detect the presence either of M or of C. Table II shows that pepsin did not digest C during a 4 day

exposure, although the enzyme had not lost its activity, as proved by complete digestion of M added on the 3rd day of the experiment. The same kind of result is shown for the tryptic digestion experiment in the lower part of Table II. The trypsin had lost some of its activity in the course of the experiment, as indicated by the slow rate of digestion of the M added on the 3rd day and by the failure to digest this substance completely even after 48 hours contact. The greater part of the M was, however, digested; but the C was unaffected since no difference in the precipitin tests was detected in the active and the inactivated trypsin digestion mixtures. The failure of trypsin and of pepsin to digest the C substance during a prolonged exposure is convincing evidence that this substance is not an ordinary protein, although it does not eliminate the possibility that C might be digested by some other protein enzyme. However, this resistance to digestion together with the available chemical data increases the probability that the non-type-specific C substance in this species is a carbohydrate.

No extensive direct evidence is available that C in extracts is non-antigenic. No rabbits immunized with extracts for other purposes, however, have ever produced antibodies against C.

DISCUSSION.

This discussion includes a consideration of the chemical and immunological relationships of all the antigenic substances so far identified in extracts of hemolytic streptococci, and covers the data presented in both the preceding and the present papers which have shown a complicated structure of antigens in the hemolytic streptococcus. There have been demonstrated at least two non-type-specific substances in extracts of hemolytic streptococci, one protein and one probably carbohydrate, in addition to the type-specific fraction. This type-specific fraction (M), first detected by the use of antibacterial sera absorbed with heterologous hemolytic streptococci, is undoubtedly a protein. This is shown by its method of preparation, by its high nitrogen content in the form of protein nitrogen, and by the loss of activity associated with removal of the NH_2 group. In addition, the fact that it is easily digested by trypsin in alkaline or in neutral solution and by pepsin quite definitely proves its protein nature. In view of this finding, the failure to immunize rabbits with extracts containing this

substance is perplexing. The most probable explanation of this failure seems at first that in the course of extraction the protein has been denatured to such an extent that it is no longer a functional antigen, although it is highly reactive in the precipitin test, a behavior comparable to that of the type-specific carbohydrates of other species. The possibility that this result is due in the present case to denaturation has not been disproven; but the failure to immunize other rabbits with the acetic acid precipitate of simple saline extracts of pulverized bacteria offers some evidence that denaturation is not the cause of the non-antigenicity of this protein. Precipitin tests with the extract used to immunize these animals showed that the type-specific fraction (M) was present. The non-type-specific nucleoprotein (P) was also present; and antibodies were readily produced against it. Although the question of the antigenicity of the type-specific protein fraction must be left open for further work, the evidence presented suggests that it is a haptene in Landsteiner's sense (6), capable of reacting with antibodies produced in response to injection of the bacterial cell but incapable of giving rise to antibody production after separation from its cellular complex. Anaphylactic experiments, to be reported in another paper of this series, suggest the same thing. Other bacterial haptenes have been described but they are all non-protein, for example, pneumococcus and Friedländer's bacillus specific carbohydrates. Gay and Robertson (7), however, in 1913 worked with a protein, globin, which behaved immunologically in an analogous manner. Globin alone did not produce any antibody response in rabbits although it reacted with antibodies produced on injection of the combination, globin-caseinate. It is, therefore, in the same sense a haptene.

The species-specific fraction (C), first studied by Hitchcock, seems to correspond chemically and in the characteristic physical properties of its immune precipitate to the carbohydrates which in some other species are responsible for the type-specific reactions. Attempts were made to prepare sufficient quantities of this material for adequate chemical analyses, but large amounts of bacterial extract yielded such small fractions of active substance that only meager chemical data could be obtained. Such tests as could be made indicated the probability that the so called C substance was a carbohydrate. The failure of prolonged tryptic and peptic digestion to affect the precipitin

titer of this substance supported the supposition that it was not a protein, as did its passage through parchment and through certain collodion dialyzing sacs. Precipitin tests in the present experiments confirm Hitchcock's conclusion that this substance is entirely non-type-specific in its serological reactions. It is, however, unrelated to the non-type-specific nucleoprotein, since it never reacts with antisera prepared against the nucleoprotein.

The nucleoprotein antigen (P), described more fully in an earlier paper, is even less specific than the non-type-specific carbohydrate-like C substance and shows group relationships with nucleoproteins of related Gram-positive cocci, such as the non-hemolytic streptococci, the pneumococci, and the staphylococci. While such group relationships are marked, they are not absolute. Nucleoproteins within the group of the hemolytic streptococci, on the other hand, seem identical. No attempt was made to separate the nucleoprotein into a species-specific fraction and fractions reactive with other species, although it seems possible that this could be done if the proper chemical methods were available.

The nucleoprotein, P, is a true antigen and stimulates antibody production when injected into rabbits. It differs in this respect from the other reactive fractions obtained from the hemolytic streptococcus. While insufficient material was at hand for exhaustive tests in regard to the antigenicity of the C substance, considerable indirect evidence was available that it could not induce antibody formation since the serum of no rabbit immunized with extracts of hemolytic streptococci ever showed the slightest trace of reaction with purified C. The similar lack of antibody production on injection of the type-specific protein M has already been mentioned. Thus, there are probably at least two haptenes, one apparently carbohydrate and the other protein, in the antigenic complex of the hemolytic streptococcus.

The presence of still another non-type-specific protein fraction was suggested in certain of the extracts which, except for this impurity, consisted almost exclusively of the type-specific M. After purification of M from certain strains to such an extent that it gave only type-specific reactions with most antisera, it persistently gave cross-reactions with occasional heterologous antibacterial sera, unless the sera were absorbed, as previously described, with heterologous hemolytic

streptococci (1). A study of the intensities and range of these reactions together with the fact that no reaction was obtained with potent anti-P sera strongly suggests the existence of still another non-type-specific substance. Although digestion with trypsin and with pepsin shows that it is a protein, it evidently is a different protein from the non-type-specific nucleoprotein, P Further evidence of its existence was obtained later by means of anaphylactic experiments.

The present analysis of the antigens of the hemolytic streptococcus, therefore, indicates a type-specific protein, (M), at least one non-type-specific protein (the so called nucleoprotein, P), with the possibility of another less well defined non-type-specific protein (Y), and finally a substance (C) which is probably a carbohydrate and is specific for species but not for type. The intact bacterial cell induces antibody formation against all these substances, but so far only the nucleoprotein has been found capable of eliciting antibodies after disruption of the cell body.

SUMMARY.

1. The chemical and immunological characteristics of the species-specific substance (C) of *Streptococcus hæmolyticus* are considered.

(*a*) It seems to be a carbohydrate because considerably purified preparations of C resisted prolonged tryptic and peptic digestion and were negative for the ordinary protein color tests but gave positive Molisch reactions to the limit of the precipitin titer. One such "purified" lot, however, had 4.2 per cent nitrogen and only 28 per cent reducing sugars on hydrolysis. Whether the nitrogen was due to impurities or was combined in the C substance itself, as is true of the Type I pneumococcus specific polysaccharide, cannot be stated without more material.

(*b*) The C substance forms precipitates with antibacterial sera prepared against heterologous, as well as against homologous hemolytic streptococci. These precipitates are typical discs like those formed by type-specific carbohydrates of other species of bacteria. C does not precipitate antinucleoprotein sera.

(*c*) While there is only slight direct evidence that the C substance is not antigenic, there is considerable indirect proof that this is the case. It probably is a haptene in the sense of Landsteiner.

2. A discussion is included of the chemical and immunological relationships of all the serologically active substances so far identified in extracts of the hemolytic streptococcus.

BIBLIOGRAPHY.

1. Lancefield, R. C., *J. Exp. Med.*, 1925, xlii, 397; 1928, xlvii, 91, 469.
2. Hitchcock, C. H., *J. Exp. Med.*, 1924, xl, 445.
3. Heidelberger, M., and Avery, O. T., *J. Exp. Med.*, 1923, xxxviii, 73; 1924, xl, 301.
4. Heidelberger, M., Goebel, W. F., and Avery, O. T., *J. Exp. Med.*, 1925, xlii, 727.
5. Avery, O. T., personal communication. Lancefield, R. C., *J. Exp. Med.*, 1925, xlii, 377. Julianelle, L. A., *J. Exp. Med.*, 1926, xliv, 735.
6. Landsteiner, K., *Biochem. Z.*, 1919, xciii, 106. Landsteiner, K., and Simms, S., *J. Exp. Med.*, 1923, xxxviii, 127.
7. Gay, F. P., and Robertson, T. B., *J. Exp. Med.*, 1913, xvii, 535.